PHARMACOLOGY OF A NOVEL CLASS OF ALLOSTERIC MODULATORS FOR THE ALPHA4 BETA2 SUB-TYPE OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

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A

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of a large family of ligand gated ion channels that mediate inhibitory and excitatory neural transmission in the central nervous system (CNS). The nicotinic subfamily has been implicated in a range of neurological disorders including autism, Alzheimer's disease and nicotine addiction; diseases that are currently both challenging and costly to treat. Despite the apparent importance of nAChRs in these disorders, only a limited number of drugs are currently available for altering nicotinic signalling in the CNS. No drug therapies are currently available that specifically target autism and only a limited number of drugs are available for the treatment of Alzheimer's disease. This thesis presents a novel class of nAChR ligands based on the natural product desformylflustrabromine (dFBr).

Desformylflustrabromine (dFBr), a metabolite of the marine bryozoan *Flustra Foliacea,* was previously identified as an allosteric modulator of the α4β2 subtype of nAChRs. In collaboration with Dr. Richard Glennon at the Virginia Commonwealth University, College of Pharmacy, we developed a synthetic dFBr and evaluated its interaction with two of the most common subtypes of nAChRS, α7 and α4β2 (Chapter 2). We confirmed that dFBr is the active component of *Flustra Foliacea* and identified an additional inhibitory action that becomes evident as dFBr concentrations are increased beyond 10μM. This inhibition was not previously reported. Synthetic dFBr appears significantly more potent at potentiation of α4β2 receptors then reported for the natural extract and shows only inhibitory action on α7 receptors.

Multiple analogues of dFBr were designed and synthesized to determine the structure activity relation (SAR) for dFBr's action on α4β2 receptors (Chapter 3). We identified three analogues capable of potentiating responses of acetylcholine. The majority of compounds inhibited responses on both α4β2 and α7 receptors. The data presented here provide important information for determining a preliminary pharmacophore for dFBr and provide direction for the design of additional analogues on the path to development of more potent and potentially therapeutically useful analogues.

To better understand the relationship of dFBr to other nAChR modulators, we also compared the action of dFBr to that of physostigmine, zinc and 17-β-estradiol (Chapter 4). These compounds are thought to act at three different binding sites on nAChRs. All three compounds increase responses of α4β2 receptors to acetylcholine. Our data show that dFBr is distinct from the clinically used modulator physostigmine but suggests similarities in

mechanism with zinc and 17-β-estradiol. These data provide important information regarding the mechanism of dFBr modulation and provide direction for future site directed mutagenesis studies that will identify the dFBr binding site. Identification of the binding site is critical for the development of receptor models that will facilitate computer assisted drug design.

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$(\alpha 4)_2(\beta 2)_3$ – Stoichiometry of α4β2 receptors containing two α4 subunits and three β2 subunit

$(\alpha 4)_3(\beta 2)_2$ – Stoichiometry of α4β2 receptors containing three α4 subunits and two β2 subunit

17-BE – 17- Beta Estradiol

5HT – Serotonin

$5HT_3R$ – Serotonin type three receptors

A^0 – Armstrong unit

AChBP – Acetylcholine binding protein

AD – Alzheimer's disease

ADNFLE – Autosomal dominant nocturnal frontal lobe epilepsy

ANOVA – Analysis of Variation

APL – Allosteric potentiating ligands

ASM – Alanine scanning mutagenesis

ATP – Adenosine Tri Phosphate

Aβ 1-42 – beta amyloid peptide 1-42

BDZ – Benzodiazepines

CA1, CA2 and CA3 – Cornu Ammonis areas one, two and three of the hippocampus

CNS – Central Nervous System

dFBr – Desformylflustrabromine

DMSO – Dimethyl sulfoxide

EC_{50} – Half maximum effective concentration

EPSP – Excitatory postsynaptic potential

FDA – Food Drug Administration

GABA – Gama-aminobutyric acid

GABA (A) and (C) receptors – Gama-aminobutyric acid receptors type (A) and type (C)

HCl salt – Hydrochloric acid salt

IC_{50} – Half maximum inhibitory concentration

IPSP – Inhibitory postsynaptic potential

LGIC – Ligand gated ion channels

mM – Millimolar

mV – Millivolt

MΩ – Megaohms

NAc – Nucleus accumbens

nAChR – Nicotinic Acetylcholine Receptors

NAM – Negative Allosteric Modulators

NCA – Non competitive agonist

ng – Nanogram

nM – Nanomolar

NRF-1 and NRF-2 – Nuclear respiratory factor type one and type two

NUGEMPS - Nuclear genes encoding mitochondrial proteins

PAM – Positive Allosteric Modulators

S.E.M – Standard Error of the Mean

SAR – Structure Activity Relation

SCAM – Substituted Cysteine Accessibility Method

TEVC – Two Electrode Voltage Clamp

VTA – Ventral Tegmental Area

α4β2 – alpha4 beta2 subtype of nicotinic receptors

α7 – alpha7 subtype of nicotinic receptors

α-BTX – alpha Bungarotoxin

μL – Microlitre

μM – Micromolar

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CHAPTER 1: INTRODUCTION

Maintenance of homeostasis is a vital biological function. Ion channels, pore forming proteins embedded in cell membranes, regulate the flow of ions in and out of cells and maintain ionic concentrations thus playing a major role in the regulation of water and ion homeostasis inside the cell.

Ion channels conduct ions actively and passively in neurons, the specialized cells of the nervous system. Any disruption of the function of ion channels can lead to malfunctions in the nervous system, which hampers the ability of an organism to sense and react to its environment. Ion channels also play a critical role in non-neuronal systems including the cardiovascular system, renal physiological and neuro-endocrine systems.

1.1 Historical perspective:

The concept of ion channels emerged in the 1800s when Luigi Galvani published his observations that electric current could cause twitching in the legs of a dead frog. Based on these and other observations, Julius Bernstein later provided a physical and chemical explanation for all of life's processes. Bernstein hypothesized that a semi-permeable membrane surrounded nerve and muscle cells and that differences in potassium ion (K^+) concentrations inside and outside the cell explained the electrophysiological evidence of ion conductance and accounted for the phenomenon of membrane potential (i.e. membrane voltage). In 1937, electrophysiologists for the first time used the squid neurons to study ion currents. These "giant squid" axons were approximately 100 to 1000 times larger in diameter than neurons from other organisms and facilitated the study of ion conductance. In 1952, Hodgkin, Huxley and Katz used the giant squid axon as an experimental model to show that Na^+ and K^+ ions move across the neuronal membrane to produce currents responsible for generating the action potential (Hodgkin et al., 1952).

1.1.1 History of nicotinic acetylcholine receptors:

David Nachmansohn first postulated that Nicotinic acetylcholine receptors (nAChRs) were proteins present on the cell membrane (Nachmansohn, 1959). In 1969, Changeux, Kasai and Lee characterized acetylcholine receptors from the electric organ of *Eloctrophorus Electricus* using the polypeptide α-Bungarotoxin obtained from the venom of the snake *Bungarus multicinctus* (Changeux et al., 1970). Electric organs from electric eels and rays (*Torpedo Californica* and *Torpedo Marmorata)* provided a rich source of nAChRs similar to nAChRs found in muscle. The first affinity labelling of nAChRs were done on the electric organ of *Eloctrophorus Electricus* (Silman and Karlin, 1969). The receptors thus obtained were named as the α1 subunit of muscle type of nAChRs (Karlin and Cowburn, 1973). Since then, electric organ nAChRs have provided the prototypical model for other nAChR subtypes.

1.2 Classification and characteristics of ligand gated ion channels:

Ligand gated ion channels (LGIC) consist of 3 super-families:

a) Cysteine - loop ligand gated ion channel superfamily;

b) Ionotropic glutamate receptor superfamily;

c) Adenosine Tri Phosphate (ATP) - gated receptor superfamily.

The three receptor super-families are classified based on their structure, function and primary sequence. General similarities between the three LGIC super-families (Le Novere and Changeux, 2001) include their location in the cell membrane, and the presence of three major domains structural domains. They share a large extracellular domain involved in ligand recognition, a transmembrane domain which forms an aqueous pore and allows the passage of ions and an intracellular domain responsible for the communication with the intracellular signalling proteins (North, 1995). Super-families differ from one another in broadly divergent primary sequences as well as significantly different tertiary and quaternary structures. These properties are well conserved within each superfamily although ligand

selectivity and ion selectivity differ widely between individual members. Super-families are further subdivided based on the endogenous agonist thought to activate the receptor.

1.3 Classification of Cysteine-loop ligand gated ion channels:

The Cysteine-loop or "Cys-loop" superfamily is characterized by several unique structural features (Arneric and Brioni, 1999). A signature 15 amino acid spaced disulphide loop between two cysteine residues located in the extracellular domain of the receptor is conserved in all members of this superfamily (Arneric and Brioni, 1999). The superfamily is also characterized by a tertiary structure consisting of five individual subunits with agonist binding sites thought to form at the subunit interfaces (Arneric and Brioni, 1999). An opening at the center and a regulated ion channel form the pore of all cys-loop receptors and an intracellular "vestibule" is formed by the intracellular domain that regulates the ion conductance (North, 1995). Individual members of the cys-loop superfamily respond to four different endogenous neurotransmitters thus forming four sub-families. Cys-loop receptors respond to the neurotransmitters acetylcholine (ACh), gama-aminobutyric acid (GABA), serotonin (5-HT) and glycine (Connolly and Wafford, 2004) although these ligands also activate a family of G-protein linked receptors (Arneric and Brioni, 1999). Table 1.1 identifies the different Cys-loop LGIC subtypes and subunits. The major classes of Cys-loop LGIC receptors are:

a) Muscle and neuronal nAChRs.

b) GABA (A) and (C) receptors.

c) Serotonin type 3 receptors ($5HT_3R$).

d) Glycine receptors

The acetylcholine receptors and the ($5HT_3R$) are non-selective cation conducting (K^+, Ca^{++}, Na^{++}) receptor channels (North, 1995), while the GABA (A) and (C) receptors and the glycine receptors are non-selective anion conducting (e.g. Cl^-) receptor channels (North, 1995)

conducting receptors have depolarizing, excitatory effects while anion conducting receptors are hyper-polarizing and inhibitory.

GABA receptors are divided into types A, B and C. GABA (B) receptors is a group of G-protein coupled receptors while types A and C are ligand gated ion channels (Enna and Möhler, 2007). Sixteen different subunits compose pentameric GABA (A) receptors (α1-6, β1-3, γ1-3, δ, ε, π and θ subunits) (Olsen and Sieghart, 2008) and three different subunits (ρ1-3) form pentameric GABA (C) receptors (Bormann, 2000). The Serotonin type 3 receptors ($5HT_3R$) are composed of 5 different subunits ranging from A to E. $5HT_3R$ (A) receptors are homomeric and contain only the A subunit. $5HT_3R$ A subunits can also combine with $5HT_3R$ B subunits to form hetero pentameric receptors, the stoichiometry of which is unknown (Davies et al., 1999). While the genes for $5HT_3R$ (C), $5HT_3R$ (D) and $5HT_3R$ (E) have been described, their functional characteristics have not been identified (Niesler et al., 2003). Glycine receptors can be composed of pentameric arrangements of α1-4 or a β subunit (Betz et al., 1999). Strychnine sensitive glycine receptors express differently in human embryos versus adults. Pre-natal glycine receptors are mainly homomers consisting of α2 subunit while the adult glycine receptors mainly consist of heteromeric α1β subunit with a stoichiometry of 3:2 or 4:1 of α1:β (Webb and Lynch, 2007).

Table1.1: Classification of Cys-loop ligand gated ion channels (North, 1995):

Neurotransmitter	**Receptor subtypes and subunits**
Acetylcholine	Muscle: α1, β1, γ, δ & ε
	Neuronal: α2, α3, α4, α5, α6, α7, α8, α9, α10. β2, β3 & β4
Serotonin (5-HT)	Subtype: A, B, C, D & E
Gamma amino butyric acid	Type A: α1-6, β1-3, γ1-3, δ, ε, π & θ
(GABA)	Type C: p1, p2 & p3
Glycine	Subtype: α1, α2, α3, α4 & β

1.4 Classification of nicotinic receptor subunits and nomenclature:

Nicotinic acetylcholine receptors (nAChR) represent a large and diverse subfamily of Cys-loop LGIC receptors. These receptors are characterized as "nicotinic acetylcholine receptors" because all members of this group respond to exogenously applied nicotine and to the endogenous neurotransmitter ACh (Arneric and Brioni, 1999). The designation "nicotinic" reflects the importance of nicotine as an agonist and pathological agent at nAChRs and further distinguishes between the nicotine insensitive and acetylcholine sensitive metabotropic muscarinic receptor family (Clementi et al., 2000). The muscarinic receptors are composed of seven transmembrane domains and are distinct from the nAChR family of LGICs (Caulfield and Birdsall, 1998).

As described above, membranes of electric rays (*Torpedo marmorata*) are a rich source of a nAChR that are similar in structure to nAChRs found in muscle. Using photo-affinity labeling studies, four subunits of torpedo nicotinic receptors were identified and classified as α, β, γ and δ in order of increasing molecular weight (Lindstrom et al., 1995). These four muscle subtypes of nAChRs were subsequently found to be very similar to the nAChRs obtained from membranes of electric rays. Hence, the four muscle subunits were also named α, β, γ and δ in order of increasing molecular weight (Lindstrom et al., 1995).

The cloning and purification of different neuronal subunits of nAChRs were pursued simultaneously with muscle nAChRs. It determined that the muscle α subunit contained a pair of Cysteine residues in position 192 and 193 (Silman and Karlin, 1969). This pair of cysteines is different than those that characterize the cys-loop family and are contained only in nicotinic subunits that bind nicotinic agonists. Nicotinic subunits later identified as containing a homologous pair of Cysteine residues were all named α subunits. Using photo-affinity labelling, these two adjacent Cysteine residues were shown to be in the vicinity of the ACh binding site in the muscle α subunit and have been determined to be a key feature of the ACh binding site in all α-subunits (Clementi et al., 2000). Nine other α subunits forming

nAChRs receptors have been discovered. Based on the order in which they were discovered, these nine subunits were classified as α2 to α10 with muscle type receptors containing only the α1 subunit and neuronal receptors containing different combinations of α2-α10 (Arneric and Brioni, 1999).

Homologous neuronal subunits lacking the two Cysteine residues near the ACh binding sites were called β subunits (Arneric and Brioni, 1999). β subunits were unable to bind to photo-affinity labels suggesting the absence of ACh binding sites on these subunits (Arneric and Brioni, 1999).

The nomenclature of nAChR has now been standardized as shown in Figure 1.1. The muscle subtype of nAChRs consists of α1, β1, γ, δ and ε subunits. Twelve neuronal subtypes of nAChRs have been identified: α-2 through α-10 and β-2, 3 and 4 subunits (Lukas et al., 1999).

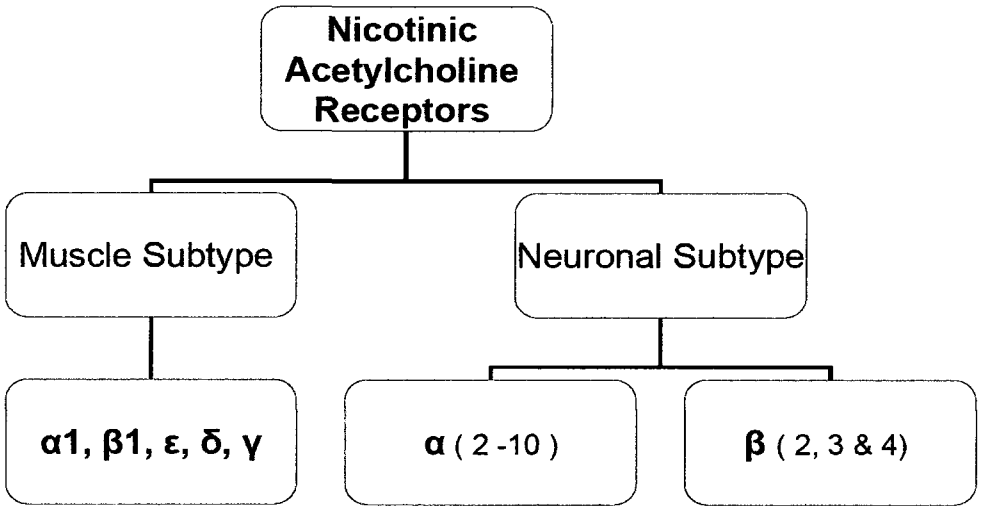

Figure 1.1: Classification of nicotinic acetylcholine receptors.

1.5 Nicotinic receptor subtypes:

Muscle nAChRs consist of fetal and adult subtypes: The fetal subtype is composed of two subunits of α1 and one subunit each of β1, γ and δ. The δ-subunit is substituted by the ε-subunits in the adult subtype. Neuronal nAChRs are composed of pentameric arrangements of a wide variety of combinations of α-1 through α-10 and β-2, 3 and 4 subunits thus the expression of neuronal nAChRs is complex.

α7 subunits appear to express primarily as homo-pentameric receptors in the brain (McGehee and Role, 1995) although α7 subunits can also form functional heteromeric receptors in combination with the β2 subunit in vitro when expressed in *Xenopus* oocytes (Khiroug et al., 2002).. The α8 and α9 subunits also express as homo-pentameric receptors although α7 can form hetero-pentamers with α8 and α9 subunits can combine with α10 (Arias, 2000) (Gotti and Clementi, 2004). α10 subunits have only been identified to form heteromers with α9 subunits and have not been identified as homomers or in heteromeric combinations with any other nAChR subunits (Gotti and Clementi, 2004). Receptors containing α7 and α8 or α9 and α10 are the only heteromeric neuronal subtypes of nicotinic receptor which bind α-bungarotoxin (Clementi et al., 2000).

Neuronal nAChR subunits α2, α3, α4 and α6 can combine with β2 and β4 subunits to form receptors that are composed of pentameric arrangements of two different subunits (Lukas et al., 1999). Combinations of α2β2, α2β4, α3β2, α3β4, α4β2, α4β4, α6β2 and α6β4 have been identified [1] (Gotti et al., 1997). The α4β2 receptor has been shown to express in two different stoichiometries of $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ with the high ACh affinity receptor

[1] Note on nicotinic receptor nomenclature: The number following each subunit identifier (non-subscripted) is part of the subunit name – ex. α4 refers to the α-subunit classified as neuronal α subunit number 4. The subscript identifies the number of specific subunits that comprise the pentameric receptor. Hence, a designation of α4β2 simply indicates the pentamer is composed of α4 and β2 subunits but the stoichiometry is undefined; usually referring to a heterologous mixture of stoichiometries. A designation of $(\alpha4)_3(\beta2)_2$ defines the stoichiometry and identifies the receptor as comprised of 3 α4 subunits and 2 β2 subunits. The precise arrangement of these subunits relative to one another is ambiguous and generally unknown.

predominating in brain (Gotti et al., 2007). $(\alpha4)_2(\beta2)_3$ receptors bind ACh with high affinity of about 2 μM, while $(\alpha4)_3(\beta2)_2$ receptors bind ACh with low affinity of about 70 μM (Zwart and Vijverberg, 1998). α5 and β3 do not form binary complexes or homomers but form ternary complexes with α2, α3, α4, α6, β2 and β4 in various permutations and combinations (e.g. α4β2α5) (Clementi et al., 2000). Additionally a quaternary complex of subunits could be formed through combination of α5 and β3 subunits and the α2, α3, α4, α6, β2 and β4 subunits (e.g. α3β2β4α5) (Vernallis et al., 1993). Complex combinations having more than one α subunit and more than one ACh binding site (e.g. α3β2α6β2β4) are also possible (Clementi et al., 2000). Different ACh binding sites are formed between the α1γ, α1δ and α1ε subunits of muscle nicotinic receptors. Gating properties, ligand selectivity and modulation of nAChR subtypes are dependant on subunit composition (Clementi et al., 2000). Even though a subunit in a receptor may not contribute to the agonist binding site it will contribute to the ion pore and conformational stability of the receptor (Clementi et al., 2000).

1.6 Distribution of neuronal nAChRs:

Peripheral Tissues:

The tissue distribution of nAChRs in mouse has been well mapped using techniques such as *in situ* hybridization and immuno-histochemistry (Figure 1.3). The expression of muscle nicotinic receptors is found in other locations including the thymus where they are thought to modulate immune responses (Bruno et al., 2004). The expression of functional α7 nAChRs was also found in developing mammalian muscle cells (Fischer et al., 1999), although expression decreased in adults. Additionally, mRNA expression of α4, α5, β2 and β4 nicotinic subunits have been found in vertebrate muscle cells (Corriveau et al., 1995). The role of neuronal receptors in muscle tissue has not been conclusively determined but they may control metabolic and trophic functions as well as gene expression by modulating calcium influx. Nicotine binding sites have also been identified in B-lymphocytes and in

circulating T-lymphocytes (Paldi-Haris et al., 1990). Human macrophages express α7 receptors and the activation of these receptors by nicotine decreases the release of tissue necrotic factor – α (TNF- α) and interleukins 1 and 6 induced by endotoxin polysaccharides (Wang et al., 2003). Mice alveolar macrophages express α4β2 but not α7 receptors (Matsunaga et al., 2001). The presence of nAChR α3, α7, α9, β2 and β4 subunits has been demonstrated in human epidermal cells (Grando, 2001). Vascular endothelial cells of blood vessels also contain many nicotinic receptor subunits including the α3, α5, α7, α10, β2 and β4 subunits (Macklin et al., 1998). Vascular smooth muscles express the α2, α3, α4, α5, α7 and α10 subunits (Bruggmann et al., 2003). Endothelial cells of the blood brain barrier express α5, α7, β2 and β3 subunits (Abbruscato et al., 2002). Nicotine is known to alter the permeability of the blood brain barrier, possibly by reducing the expression of the α7 and β2 subunits and the tight junctional protein ZO-1 (Abbruscato et al., 2002).

The Central Nervous System:

The mapping of nAChRs in the human brain is much less comprehensive than for rat and mouse brains which have been used to study nAChR distribution (Gotti et al., 2006). A variety of techniques have been used to study the localization of nAChR in the brain including in-situ hybridization, immuno-histochemistry and radioligand binding studies using ligands such as nicotinic and α-bungarotoxin, nicotinic receptor gene knockout studies and positron emission tomography.

The majority of nAChRs in the brain can be divided in to two general categories: receptors that bind ACh with high affinity (nM concentrations) and those that bind with low affinity (μM concentration). Low affinity ACh receptors are typically the homomeric α7 nAChRs which also bind to α-bungarotoxin. High ACh affinity receptors are typically the α4β2 receptors which do not bind to α-bungarotoxin. High affinity α4β2 receptors account for about 90% of high ACh affinity receptor in the brain (Whiting and Lindstrom, 1986).

Dopaminergic neurons in the mesocorticolimbic pathway mediate the addictive property of nicotine and α4β2 receptors are implicated in this process (Picciotto et al., 1998). The nucleus accumbens and prefrontal cortex receive projections of these dopaminergic neurons from the ventral tegmental area. Almost all dopaminergic neurons in the ventral tegmental area express α4 and β2 receptors (Klink et al., 2001). The Substantia nigra pars compacta contains moderate to high levels of α4β2 nAChR mRNA (Wada et al., 1989). Immuno-electron microscopy shows the presence of the β2 subunit in the pre-synaptic region of dopaminergic axons in the dorsal striatum (Jones et al., 2001). The presence of the α4 subunit has also been demonstrated by immuno-precipitation in dopaminergic projections of the dorsal striatum (Zoli et al., 2002).

The distribution pattern of α4 and β2 mRNA is well characterized in the primate (monkey) brain (Quik et al., 2000). The most intense expression of these two nAChR subunits is seen in the dentate gyrus of the hippocampus, cerebellum, locus coeruleus, cortex and lateral geniculate nucleus (Rubboli et al., 1994). α4 and β2 mRNA have moderate levels of expression in the CA regions of the hippocampus, hypothalamus and thalamus. Expression is lowest in regions such as the caudate nucleus and locus coeruleus (Rubboli et al., 1994). While there is some evidence that nicotinic receptor signalling can be post-synaptic (Roerig et al., 1997), most of the nicotinic receptor signalling seems to be pre-synaptic in the brain (Gray et al., 1996). Immuno-histochemistry and direct electrophysiological experiments have confirmed the pre-synaptic location of neuronal nicotinic receptors. In one experiment, it was demonstrated that nicotine induced inward currents in large pre-synaptic calyces that were blocked by α-BTX, suggesting the involvement of pre-synaptic α7 nAChR (Coggan et al., 1997). Therefore it is proposed that nAChRs in the CNS primarily modulate rather than directly mediate synaptic transmission (Gray et al., 1996) The pattern of expression of the α7 receptor subunit is widespread and has considerable overlap with the expression of α4 subunit mRNA (Quik et al., 2000).

The expression of α7 receptors is highest in the dentate gyrus of the hippocampus. The beta-amyloid (a-β1-42) peptide binds with high affinity to the α7 receptors expressed pre-synaptically in the hippocampal neurons of patients with early stage Alzheimer's disease (Nagele et al., 2002). A strong to moderate expression of α7 is seen in the CA2 and CA3 regions of the hippocampus, cerebellum, and reticular nucleus of the thalamus (Figure 1.2). Moderate to low expression is observed in CA1, the lateral geniculate nucleus, and other thalamic nuclei, cortex, subthalamic nucleus, locus coeruleus, and substantia nigra (Hellstrom-Lindahl et al., 1999).

1.7 Structure of Cysteine-loop ligand gated ion channels:

1.7.1 General structure of 'Cys-loop' ligand gated ion channels:

Cys-loop ligand-gated ion channels play an important role in electrochemical neuro-transmission throughout the nervous system and other tissues of the body. Embedded in the cell membrane, these receptors are composed of five similar (homopentameric) and five different (heteropentameric) polypeptides. The representation of single subunit is depicted in Figure 1.3. The five subunits are arranged around an axis perpendicular to the cell membrane forming a pore that conducts ions from the outside to the inside of the cell. A single subunit typically contains anywhere from 400 to 700 amino acids. The amino acid sequence of every subunit is largely conserved among all members of the Cys-loop superfamily, and is presumed to be structurally similar. Three structurally distinct domains are observed in subunit tertiary structure:

The extracellular ligand binding domain: The N-terminal hydrophilic extracellular region forms the ligand binding domain in Cys-loop ligand-gated ion channels and contains a 15-amino acid spaced disulphide bond between the two Cysteine residues (Arias, 2000). The crystalline structure of the torpedo nicotinic receptor has been resolved up to 4 Armstrong

units (A^0) using electron diffraction (Unwin, 2005) and optimized by molecular modelling. The resultant structure shows the amino terminal of each subunit with an N-terminal α-helix, two short 3_{10} helices and a core of ten β-sheets which forms a "sandwich" and houses the actual ligand binding site. The ligand binding site is located between the two adjacent subunits, about 35 A^0 from the membrane. Figure 1.5 shows the ribbon structure of the refined model of the nAChRs obtained by Unwin *et. al.* Loops β1-β2, Cys-Cys and β8-β9 are essential for coupling agonist binding to channel gating (Bouzat et al., 2004).

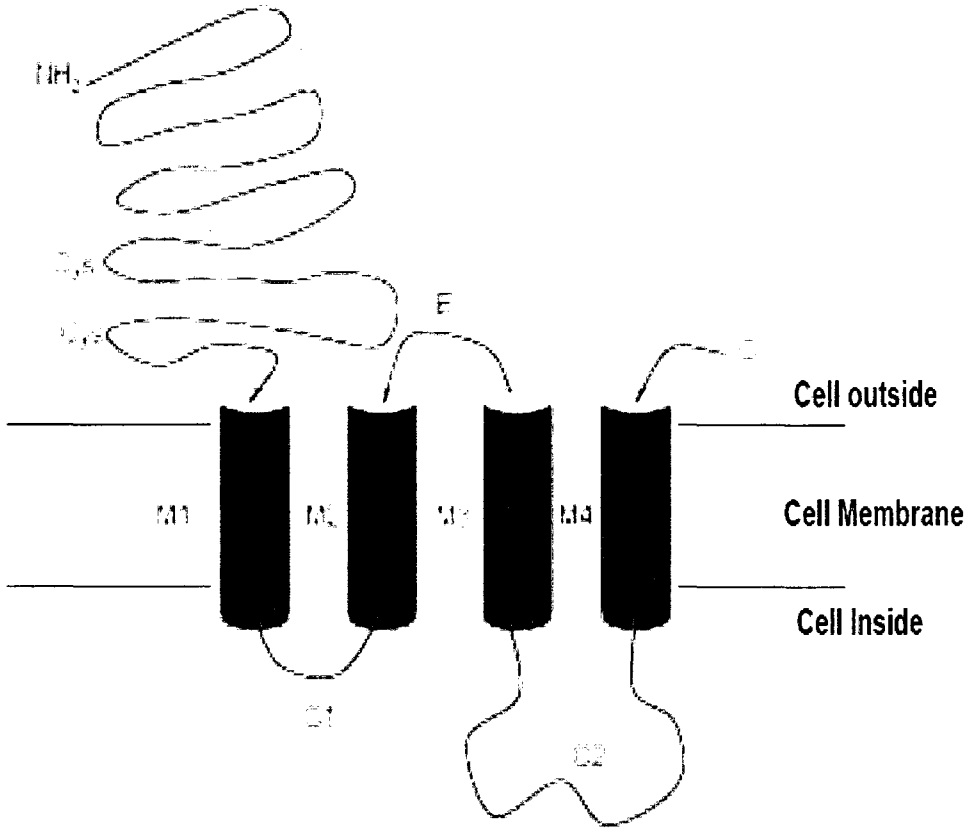

Figure 1.2: Representative structure of the ligand gated ion channel subunit. NH_2 – N terminal of the protein, C1 – small intracellular loop between M1 and M2 domain M1, M2, M3, M4 – 4 transmembrane domains, C2 – Variable intra cellular loop.

The transmembrane domain: The amino terminal sequence that forms the extracellular domain is followed by four highly hydrophobic α-helices M1, M2, M3 and M4 that comprise the transmembrane domain. The M2 transmembrane segment lines the pore of the ion channel. Five M2 segments, one from each of the five subunits composing the pentameric receptor, constitute the channel pore. Each M2 segment is surrounded by three other transmembrane segments (M1, M3 and M4). In a pentameric receptor the five central M2 segments are surrounded by 15 hydrophobic α-helices. A kink in the M2 helix produces a constriction near the mid-point of the channel due to interactions with the surrounding hydrophobic residues of the neighbouring M1, M3 and M4 domains (Unwin, 1993a). The M1, M3 and M4 transmembrane helices are in contact with the surrounding lipids of the cell membrane. The interaction of lipids with the transmembrane domains also contributes to the kinetics of channel gating in ligand gated ion channels (Spitzmaul et al., 2004). A tight hydrophobic narrowing of the channel in the middle of the membrane that creates an energetic barrier for ions and the kink at the centre of the M2 domain are thought to serve as the channel gate (Miyazawa et al., 1999). Photo-affinity labelling, site directed mutagenesis and biophysical methods have been used to deduce the structure of the ion conducting pore. Structural studies have identified the presence of conserved Leucine residues in the middle of the M2 domain (Miyazawa et al., 2003). This Leucine along with a nearby valine residue forms a hydrophobic girdle that makes up the channel gate. At its narrowest point the channel opening is approximately 3.5 A^0. This is smaller than the size of a K^+ or Na^+ ion with its single hydration shell. The hydrophobic girdles in the channel counter the electrostatic stabilization and release the ion from its shell of hydration to permit ion conductance. The hydrophobic interactions between the amino acid residues forming the gate are sufficiently weak to destabilize upon agonist binding, leading to channel gating and re-stabilization upon the loss of agonist from the ligand binding site (Unwin, 1993b).

The intracellular cytoplasmic domain: The intracellular cytoplasmic domain is hydrophilic and mostly α-helical in structure. The intracellular loop is located between the M3 and M4 domains. This is a highly variable region of the protein and its length differs widely between subunits. It varies in size from 30 to 200 amino acids in length. Electron microscopy of nicotinic receptors has shown that the cytoplasmic loop forms a closed cage with openings that presumably allow the passage of ions into the cell from the pore of the channel (Miyazawa et al., 1999). It is likely that charged amino acid residues located on the inner surface of the cytoplasmic loop of the pore regulate the passage of ions in the cell (Miyazawa et al., 1999). In the 5-HT_3R a region of the cytoplasmic loop called the HA-stretch has been identified as a major determinant of channel conductance (Kelley et al., 2003). The same study identified three positively charged Arginine residues in this region which strongly influence channel conductance in the 5-HT_3R. The region where the three Arginine residues are located is predicted to be an amphipathic helix in the intracellular loop (Miyazawa et al., 1999). Mutation in this amphipathic helix in the ε subunit of the muscle receptor leads to congenital myasthenic disease due to changes in gating kinetics of the receptor (Wang et al., 2000a). Structural determination techniques such as electron diffraction place these residues on the outer rim of the opening present at where cytoplasmic regions of adjacent subunits meet (Miyazawa et al., 1999).

The intracellular loop also contains multiple phosphorylation sites that could potentially modulate ion channel conductance (Swope et al., 1999). The currents induced by 5HT binding to the 5-HT_3R are potentiated in the presence of protein kinase C and casein kinase (Hubbard et al., 2000). This action is presumed to occur due to the phosphorylation of specific amino acids of the intracellular loop. An increase in conductance at a single channel level is also observed due to the phosphorylation of amino acids in the intracellular loop of the 5-HT_3R (Van Hooft and Vijverberg, 1995). The intracellular domain is also responsible for clustering and anchoring these receptors in the cell membrane.

Figure 1.3: General structure of nicotinic receptor. From Protein Data Bank (PDB) file 2BG9 (Unwin, 2005). Side view as seen using DeepView Swiss Pdb Viewer version 3.7.

The C-terminal of the Cys-loop ligand gated ion channels is located extracellular following the M4 loop. The C-terminal is short and presumed to contain a binding site for steroids that modulate the channel. The C-terminal of the α4 subunit of nAChRs has been identified as the binding site responsible for the potentiating action of 17-β-estradiol on α4β2 subtype (Paradiso et al., 2001).

1.7.2 Structure of ligand binding site of nAChRs:

Electron diffraction has been utilized to identify the detailed crystalline structure of *Torpedo* nAChRs to a resolution of 4 A^0 (Unwin, 2005). The discovery of acetylcholine binding protein (AChBP) and its subsequent crystallization (Brejc et al., 2002) has been used to optimize the electron diffractions structures of the N-terminal ligand binding domain of nAChRs. The currently accepted structure of nAChRs has been developed from a combination of techniques including electron diffraction, homology modelling, and models of ligand docking (Arias, 1997). Site directed mutagenesis, substituted cysteine accessibility studies and radioligand binding have enabled further refinement of these structures (Arias, 1997).

After it was demonstrated that nicotinic receptors were cell membrane bound proteins, it was hypothesized that the acetylcholine interaction site on these receptors would be on the extracellular portion of the protein because positively charged acetylcholine molecules could not pass through the cell membrane. Based on this hypothesis, it was experimentally shown that the acetylcholine binding sites were located near a disulphide bond in the nAChR protein which could be readily reduced (Silman and Karlin, 1969). When this disulphide bond was reduced, the receptors were found to be in a state of perpetual activation when covalently linked to bromo-acetylcholine (Silman and Karlin, 1969).

Site directed affinity labelling studies with a ligand for nAChRs demonstrated that every two moles of affinity labeled ligand coincided with one mole of receptor stoichiometrically (Weiland et al., 1976). Using the electric tissue of *electrophorus*, the nAChR subunit was identified by affinity labelling acetylcholine binding site (Reiter et al., 1972). This subunit was similar to the α1 subunit of muscle nAChRs. The α1 subunits in muscle nAChRs were then shown to contain the acetylcholine binding site for the muscle nicotinic receptor (Reynolds and Karlin, 1978). The muscle nAChR has two α1 subunits, hence it has two ACh-binding per receptor each corresponding to a singe α1 subunit. Labelling and cross-linking experiments provided evidence that the ACh-binding sites were present in the interface between the receptor subunits.

In muscle nAChRs, the α1 subunit forms the principal face of the ligand binding site. The subunit adjacent to the principle α-subunit forms the complementary face of the ligand binding site (Corringer et al., 2000). It was thought that the ligand binding site on nicotinic receptor was made by six distinct loops, named A, B, C, D, E and F. The crystal structure of the AChBP later confirmed that six discontinuous loops contribute to the acetylcholine binding site; loops A, B and C form the principal face and loops D, E and F form the complementary face (Smit et al., 2003). In the muscle nAChR the two adjacent Cysteines, Cys192 and Cys193 of the C-loop form a disulphide bond (Kao and Karlin, 1986).These two adjacent Cysteines are characteristic of all nicotinic receptor α-subunits. Additionally, four widely spaced aromatic residues of the muscle α-subunit Tyr93, Trp149, Tyr190 and Tyr198 were found to be conserved in all ACh receptor α-subunits (Galzi et al., 1990), except for neuronal α5 subunit. These aromatic amino acid residues in the α-subunit are also involved in binding ACh. These cysteines and aromatic residues, contributed by the principal face are in the Ach binding site, formed by the α-subunit. Figure 1.5 shows the ligand binding site on nAChRs.

The complementary face is formed by the subunits neighbouring the α-subunit in the muscle receptor, which also contributes to the ACh binding site. The ligand binding site is formed between the muscle α1 subunit and γ or δ subunits, but not with β subunit (Sine and Claudio, 1991). Photo-affinity labelling of the muscle nAChRs with *d*-tubocurarine specifically identified amino acids from the γ and the δ subunits involved in forming the ACh binding site. These amino acids are γ-subunit Trp53, Tyr111, Tyr117 and δ-subunit Trp55, and Arg113 (Sine, 1993). Another competitive inhibitor, benzoylbenzoylcholine, was photo-labelled to an amino acid pair on γ-subunit, Leu109 and δLeu111 (Wang et al., 2000b). The ACh binding site is located in a multi-loop structure localized in a pocket formed by aromatic residues. The electron cloud of the aromatic ring in aromatic residues present in the ACh binding site stabilizes the quaternary ammonium group of ACh by forming a cation-pi interaction (Sine, 2002). The positive charge on Arg113 forms a hydrogen bond with the oxygen in the carbonyl moiety of ACh (Arias, 2000).

The ligand binding site and the interaction of ACh with other nicotinic receptor subunits are similar to those in muscle nAChRs. The aromatic amino acid residues that characterize the binding site of muscle nAChRs are present in all of the nAChR subtypes, including the neuronal α7 and α4β2. These aromatic residues form similar interactions with acetylcholine as those found in the muscle receptor.

Figure 1.4: Ligand binding site on nicotinic receptor at the subunit interface. The marked box indicates the acetylcholine binding site on the nicotinic receptor. From Protein Data Bank (PDB) file 2BG9 (Unwin, 2005). Visualized using DeepView Swiss Pdb Viewer version 3.7.

1.7.3. Acetylcholine binding protein:

While studying synaptic modulation in snails (*Lymnelia stagnalis)*, Smit et. al discovered a protein that binds to acetylcholine (Smit et al., 2001). Purification and characterization of this protein revealed that this protein was a soluble homolog of the nAChRs. This acetylcholine binding protein (AChBP) derived from glial cells was

subsequently crystallized and its structure determined to 2.7 A^0 resolution (Brejc et al., 2002). The crystalline structure of AChBP indicates a homo-pentameric quaternary structure. Each subunit of the *Lymnelia* AChBP is composed of 210 amino acids with a sequence homology of 20-22% with nAChR subunits (Brejc et al., 2002). AChBP has also been found in California sea hare, *Aplysia californica* (Hansen et al., 2004) and *Bulinus truncates,* both invertebrates (Celie et al., 2005). The biological function of AChBP in the cholinergic synapses of snails is thought to be modulation of ACh mediated neurotransmission by binding of ACh released in the synapse. Sequence homology between the AChBP and other ligand gated ion channels suggests that structural elements of the nicotinic receptor including the Cys-loop and binding site structure are conserved. In particular, AChBP displays a significant structural similarity to the N-terminal ligand binding domain of *torpedo* nAChRs (Sine et al., 2004). Like other ligand gated ion channel receptors the ACh binding site on AChBP is also located at the subunit interface. Crystal structures of the AChBP with nicotine or carmaylcholine bound at the binding site have been obtained to 2.7 A° resolution (Celie et al., 2004).

Since the high resolution crystalline structure (see Figure 1.6) of the AChBP is available, it has been used as a template to develop homology models of the extracellular ligand binding domains of nicotinic and other LGIC receptors (Karlin, 2004). These homology models have proved useful tools for data interpretation obtained from biochemical experiments and the formulation of new hypotheses regarding LGIC binding site structure and function (Hansen and Taylor, 2007).

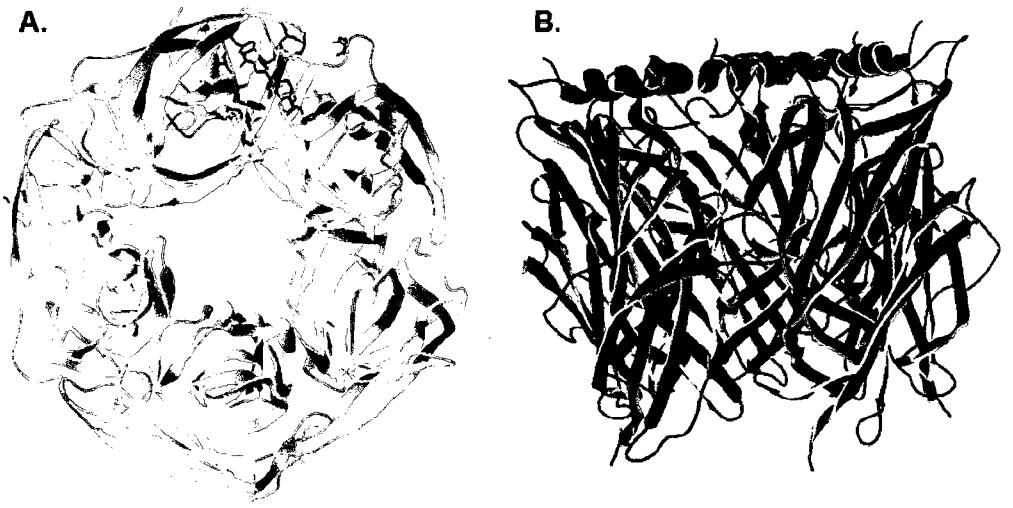

Figure 1.5: Structure of Acetylcholine binding protein. (A) Bottom view; (B) Side view. From Protein Data Bank (PDB) file 1I9B (Smit et al., 2003). Visualized using DeepView Swiss Pdb Viewer version 3.7.

1.8 Physiological role of nAChRs:

Nicotinic acetylcholine gated ion channels are primarily located at the neuromuscular junction and the neuronal synapse. The principle physiological role of nAChRs is to facilitate the process of neurotransmission although nicotinic receptors also play a role in gene transcription by permitting Ca^{2+} entry into cells.

Neuronal signalling: Under resting conditions, the membrane potential across the neuronal cell membrane is approximately -70 mV. Changes in cell membrane potential result from opening and closing of ion channels, altering the permeability of different ions (Kandel et al., 2000). The membrane potential will shift toward the Nernst potential of the most permeable ions. Hence opening of Na^+ channels leads to depolarization (increase in potential) and opening of Cl^- channels leads to hyper-polarization (decrease in potential). Acetylcholine release at the synaptic cleft leads to opening of ion channels on either a nearby pre-synaptic

or postsynaptic membranes depolarizing the membrane at that position (Kandel et al., 2000). On the pre-synaptic side, both depolarization and calcium entry serve to facilitate neurotransmitter release (Kandel et al., 2000). Post-synaptically, depolarization of the dendrites of the postsynaptic cell produces local depolarization of the membrane (Kandel et al., 2000). This depolarization slowly spreads down the dendrites toward the cell body by a process called passive conduction (Kandel et al., 2000). Typically, if only a few receptors are activated the level of depolarization is insufficient to significantly alter the potential at the neuronal cell body; however, if a larger number of receptors are open simultaneously, the cell body becomes depolarized and an action potential or active conductance is initiated at the axon hillock region of the cell and a signal is sent down the axon (Kandel et al., 2000). A single release of ACh pre-synaptically may alter the membrane potential by only +1 mV (millivolt), however a depolarization of about +15mV is typically necessary to initiate an action potential (Kandel et al., 2000). This usually involves multiple cellular inputs releasing pulses of neurotransmitter (Kandel et al., 2000). To trigger an action potential sufficient numbers of receptors must be activated within a narrow time span in a small area to produce a depolarization capable of propagating to the cell body (Kandel et al., 2000). Thus neurons integrate the incoming signal from multiple activated receptors both temporally and spatially (Kandel et al., 2000).

Synaptic potentials can be inhibitory or excitatory in nature. Inhibitory postsynaptic potential (IPSP) reduces the chance of generating action potentials by hyperpolarizing the postsynaptic membrane (Kandel et al., 2000). Excitatory postsynaptic potentials (EPSP) increases the possibility of generating action potentials by depolarizing the postsynaptic cell (Burke et al., 2001). Nicotinic receptors conduct positively charged ions, thus generating EPSP's. GABA receptors which conduct Cl^- ions hyperpolarize the membrane thus producing IPSPs and acting to prevent action potentials (Kandel et al., 2000). Thus, in addition to temporal and spatial integration of inputs, the post synaptic cell also integrates the effects of

inputs from multiple neurotransmitters (Kandel et al., 2000). Alterations in receptor numbers, conductance, open channel probabilities and ion conductivities as well as competitive binding of ligands may all contribute to changes in the delicate integration and balance of these systems. Loss of acetylcholine receptors either pre or post-synaptically will alter the signaling profile of systems mediated by nAChRs.

nAChRs in Gene expression: While the process of fast synaptic transmission is thought to be the primary neuro-physiological function of nAChRs, activation of these receptors may also alter gene expression. One example of nicotinic receptor mediated gene expression is mitochondrial biogenesis in skeletal muscle in response to vigorous exercise (see Figure 1.7). Skeletal muscles contract and relax in response to signals from motor neurons innervating the muscle cells. ACh released at the neuromuscular junction binds to the muscle nicotinic receptor on the muscle membrane surface. Since muscle nAChRs conduct calcium ions, repeated stimulation during vigorous exercise increases the concentration of calcium in the muscle cell. Elevated intracellular calcium stimulates additional calcium release from the sarcoplasmic reticulum which binds to the protein calmodulin. Calcium binding to calmodulin complex activates the Ca/Calmodulin dependent protein kinase (CaM-kinase), which translocates to the nucleus of the muscle cell and phosphorylates a transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Ojuka et al., 2003). PGC-1α has the ability to auto-regulate its own gene expression. Moreover, it induces the expression of nuclear respiratory factor-1 (NRF-1) and NRF-2 which are transcription factors for numerous nuclear genes encoding mitochondrial proteins (NUGEMPS) (Hood, 2001). The expression of mitochondrial transcription factor A (Tfam) is also induced by NRF-1 (Gordon et al., 2001). Tfam and NUGEMPS are imported into mitochondria by the protein import machinery (PIM) (Joseph et al., 2006). Tfam is responsible for regulating the expression of the 13 mitochondrial DNA (mtDNA) genes, including cytochrome *c* oxidase

subunit 1 (COX 1) (Hood et al., 2006). PGC-1α coordinates the expression of nuclear and mitochondrial genomes which ultimately leads to mitochondrial biogenesis in the muscle cell. The increase in mitochondrial density satisfies the demand for more Adenosine tri-phosphate (ATP) in the muscle cell undergoing vigorous exercise. A similar mediation of cellular processes through calcium entry may also play a role in the CNS and likely plays a role in up and down regulation of nAChR expression on exposure to nicotine. Recognition of the importance of calcium in nicotinic signalling is likely to grow as more detail about distribution and interaction of nAChRs with other neurotransmitter systems is elucidated.

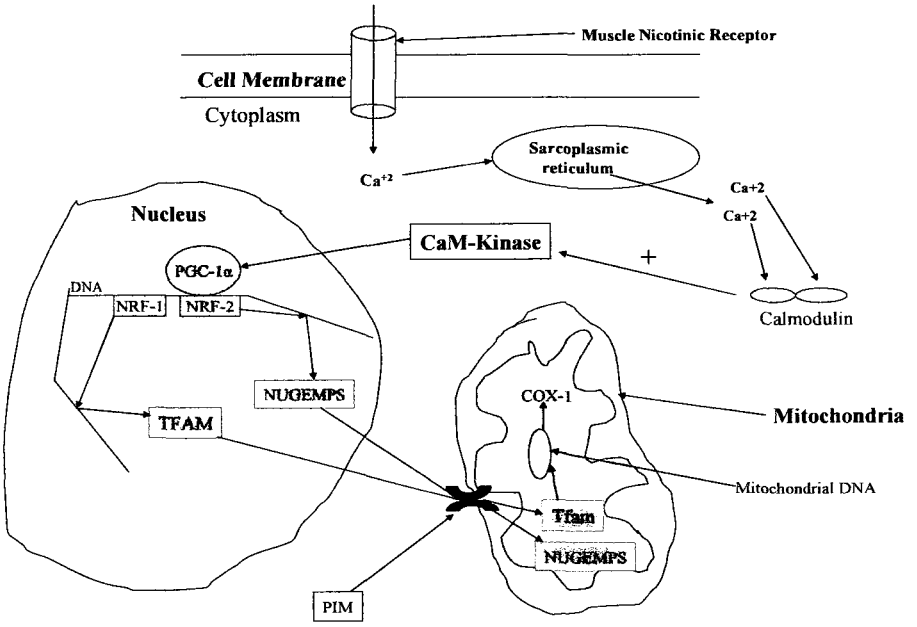

Figure 1.6: Muscle nicotinic receptor signaling causes gene transcription in the cell. CaM-Kinase – Calcium calmodulin dependent kinase, PGC-1α - proliferator-activated receptor-γ coactivator-1 alpha, NRF-1 & NRF-2 – Nuclear respiratory factor-1 and Nuclear respiratory factor-2, TFAM – mitochondrial transcription factor-A, NUGEMPS – Nuclear genes encoded mitochondrial proteins, PIM - protein import machinery, Cox-1 - cytochrome *c* oxidase subunit-1.

1.9 Functional characteristics of nAChRs:

1.9.1 The gating mechanism in nAChRs:

Gating is the process by which nAChRs open to conduct ions and close to a non-conducting or desensitized state. The gating process involves sequential changes to the stability of different confirmations or "states" of the channel. The binding of agonist to the receptor is the triggering event responsible for inducing gating in nAChRs. The bound agonist leads to conformational changes that stabilize the open channel conformation thus permitting ions to move through the pore. The ligand binding site of the nAChRs is present at the subunit interface in the extracellular domain. The ligand binding site is present in six binding loops that form the ligand-binding pocket of LGICs (Corringer et al., 2000).

Structural determinants of the gating process in nicotinic receptors: Nicotinic receptors contain a signature loop with a conserved disulphide bond in the extracellular domain. This defining structure is thought to play a significant role in the conformational changes leading to channel opening (Dougherty and Lester, 2001). In nAChRs the cys-loop is located at the bottom of the subunit interface in contact with an emergent TM2-TM2 loop. TM2 lines the channel pore and movement of this transmembrane helix is likely critical to channel opening. The interaction with the cys-loop and the TM2-TM3 loop is thought to be destabilized as a result of binding of agonists producing a rotation of TM2. A second region of the protein typically referred to as 'loop 2' is also located in a position near the membrane surface. It is possible that both the 'Cys-loop' and the 'loop 2' regions may interact with the transmembrane domain of the receptor to activate the ion channel (Brejc et al., 2001).

Electron micrograph structures of the *Torpedo* nAChR have indicated that a conserved Leucine (L251) forms a kink in the middle of the helical TM2 segment of the receptor's pore. (Unwin, 1995). The hydrophobic interactions of the leucine side chain with neighbouring amino acid side chains make a tight hydrophobic girdle around the pore

(Miyazawa et al., 2003) forming the gate of the channel. It has been proposed that the rotation of one or two TM2 segments weakens the hydrophobic interactions at the girdle, causing all of the TM2 segments to rotate and open the channel pore, allowing the passage of ions. A linear free energy relationship analysis of mutations within the TM2 segment of the nAChR δ-subunit indicates there is a conformational change of the upper half of the TM2 segment which precedes that of the lower half of TM2 (Cymes et al., 2002). This conformational change in the upper half of TM2 weakens the hydrophobic interactions, allowing the channel pore to open. The TM2-TM3 loop may thus act as a hinge that allows the opening of the channel by the rotation of the TM2 domain, since it is located on the extracellular side of the ion channel pore (Lynch et al., 1997). As described above, rotation of TM2 may be initiated by destabilization of interactions between the TM2-TM3 extracellular loop and the ligand binding domain as a result of ligand binding. This hypothesis is consistent with earlier electron micrograph structures of the nAChR (Unwin, 1995) and the close proximity of the cys-loop and loop2 with the TM2-TM3 loop. Conformational changes have been demonstrated in the TM2–TM3 loop during channel activation associated with channel gating (Lynch et al., 2001).

The molecular interactions between the extracellular domain and the TM2–TM3 loop in the nAChR are hydrophobic in nature. Miyazawa and colleagues proposed a hydrophobic 'pin and socket' interaction between a Valine residue (α1V44) in 'loop 2' of the extracellular domain and a hydrophobic pocket between residues α1S269 and α1P272 on the TM2–TM3 loop (Miyazawa et al., 2003). According to this model, binding of the ligand induces a clockwise 15° rotation of loop 2 in the α-subunit on an axis perpendicular to the membrane plane. This loop pushes the TM2–TM3 loop and rotates the TM2 transmembrane domain. This rotation causes destabilization of the hydrophobic girdle forming the channel gate in the TM2 transmembrane domain and results in the passage of ions through the channel pore (Miyazawa et al., 2003).

Mathematical modeling of channel gating: The gating of an ion channel can be represented by mathematical models which assume multiple meta-stable conformational states and channel dictating rates at which these confirmations change (Hille, 2001). A key component in modeling the gating of nAChR is the determination of the number of conformations and intermediate states of the channel (Hille, 2001). It was previously assumed that there were only three states of the receptor channel (see Figure 1.8). These were the resting state (closed), the active state (open) and the desensitized state. In the absence of an agonist, most of the channels in a population are in the resting state (closed). Ligand binding to the receptor opens the ion channel because the probability of ion channel opening increases in the presence of an agonist (Hille, 2001). During the prolonged presence of the neurotransmitter (agonist), the receptor reaches a stable, desensitized state. In the desensitized state, the channel remains closed upon application of agonist, because the agonist remains bound (Hille, 2001). The receptor recovers from desensitization by two routes. An agonist bound receptor can reopen by shifting back to the open conformation or the agonist can dissociate returning the receptor to the (closed) resting state.

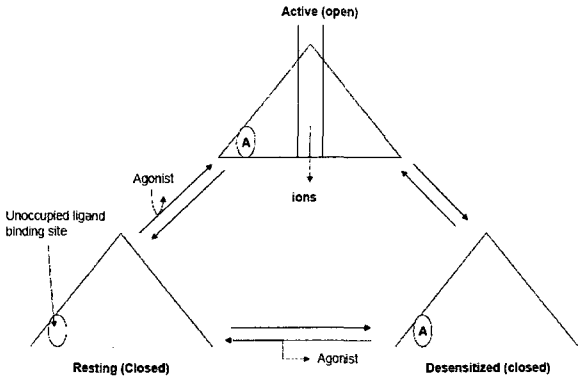

Figure 1.7: The three presumed conformational states of nicotinic receptor according to the Markov model.

The transition probabilities between the three sites are known as the Few States Model or Markov Gating Model (see Figure 1.8) This model assumes that the kinetic rate constants are independent of the time spent in the 3 states and also independent of the previous sequence of the channel state (Colquhoun and Sakmann, 1981).

1.9.2 Channel kinetics and other biophysical characteristics of nAChRs:

The nAChRs are ion channels and as such allow passage of cations into the cell. Cloned nAChRs are routinely expressed in cell lines by stable or transient transfections. Electrophysiological techniques such as patch clamp are used to study functional characteristics of nAChRs expressed in mammalian cell lines. mRNA coding for nAChRs can also be synthesized in vivo and injected into *Xenopus laevis* (frog) oocytes to study receptor function (Buckingham et al., 2006) .

Macroscopic biophysical characters of nicotinic receptor: *Xenopus* oocytes are larger in size than mammalian cells and express more nicotinic receptors numerically when injected with mRNA. Not only do *Xenopus* oocytes provide an advantage due to their size, but they also conduct a large measurable current when nAChRs are expressed in them. The nAChRs in oocytes are typically characterized using two electrode voltage clamp (TEVC) (Joshi et al., 2004). In this technique two electrodes, one corresponding to voltage and other corresponding to current, are inserted into a *Xenopus* oocyte expressing nAChRs (Joshi et al., 2004). The oocyte is continuously perfused with a buffer solution (Joshi et al., 2004) and clamped at a fixed holding potential (Joshi et al., 2004), following which the agonist is applied. Application of agonist opens the expressing nAChR channels (Buckingham et al., 2006). The current required to maintain the holding potential is recorded as the ions pass into the oocyte through the expressing ion channel (Buckingham et al., 2006). The recorded current is equivalent to the number of ions that pass through the ion channels and is a

macroscopic summation of all open nAChR channels in the oocyte. The current displays three distinct phases each corresponding to the change in the number of channels open at a particular time after exposure to agonist. Current elicited by application of agonist is plotted against time. A typical response is shown below in Figure 1.9. Shown in the figure are two traces, one obtained by application of ACh to an oocyte expressing human α7 receptors and the other from an oocyte expressing α4β2 receptors.

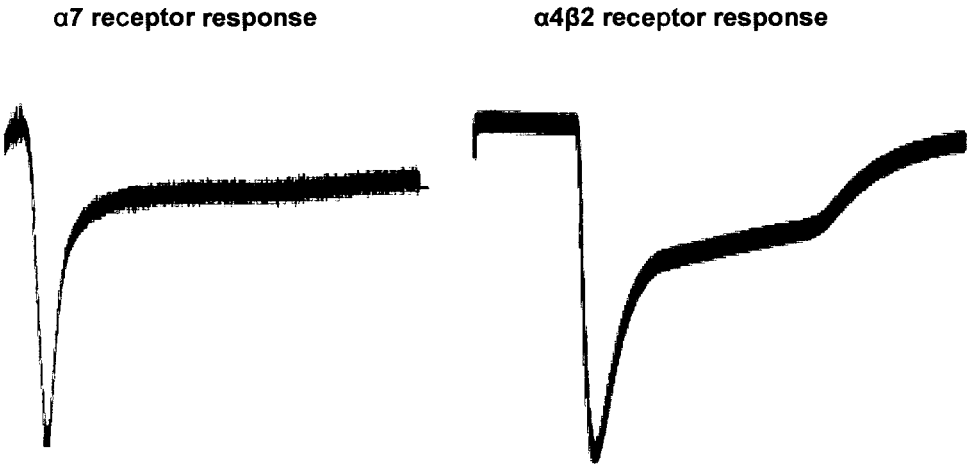

Figure 1.8: Response profiles for α7 and α4β2 receptors expressed in Xenopus oocytes. Oocytes were held at a membrane potential of -60 mV. Acetylcholine was applied to each receptor at a concentration of 100μM.

Desensitization of ligand-gated ion channel receptors: When ACh is applied at a steady state to the muscle tissue, the macroscopic end-plate potential falls within seconds via a phenomenon known as desensitization (Hatton et al., 2003). All nAChRs desensitize to some extent during prolonged exposure to agonist. This desensitization is typified by the decline in the responses shown in Figure 1.9 following the response peak. Some nAChRs like the α7 subtype have faster rates of desensitization, while others, like the α4β2 subtype, desensitize with slower rates (Kim et al., 2007). Desensitization is the result of resistance of nAChRs to

re-activation by application of agonist and is thought to be the result of the presence of a closed, ligand bound conformation of the receptor (Hille, 2001). There may, in fact, be multiple desensitized conformations of nAChRs (Raines and Krishnan, 1998). Desensitized states may offer unique drug targets for inhibitory ligands. One example of this is Sazetidine-A, a novel nAChR ligand that binds to the desensitized state of the α4β2 nAChRs without activating them (Xiao et al., 2006).

1.10 Role of neuronal nAChRs in various diseases and Conditions:

The α7 and the high affinity α4β2 receptors are the two main subtypes of nAChRs with wide distribution in the brain. As such they are involved in the patho-physiology of many diseases and disorders. Nicotine addiction, Alzheimer's disease, human epilepsy syndrome, Schizophrenia and Autism are some of the neuro-psychiatric conditions where nicotinic receptors are thought to be involved.

Nicotine addiction:

Nicotine is an agonist which binds to all subtypes of acetylcholine receptors. Obtained from the leaves of the tobacco plant *Nicotiana*, nicotine is alkaloidal in nature and highly addictive in humans. Humans primarily ingest nicotine through smoking cigarettes. The public health risk due to cigarettes and subsequent smoke, which contains nicotine, is tremendous. The addictive properties of nicotine due to self-administration are well known. Understanding the mechanism of nicotine addiction is important due to the extreme health risks associated with nicotine abuse (deRuiter and Faulkner, 2006). The effects of nicotine are complex and not well understood at the synaptic level, however the activation and desensitization by nicotine likely alters the profile of the synaptic response thus altering the temporal integration of synaptic signalling (McGehee, 2007). Synapses respond to nicotine by altering expression of receptor subtypes. The α4β2 subtype of nicotinic receptor is up-

regulated after chronic exposure to nicotine (Fenster et al., 1999). The mesolimbic dopamine (DA) projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is a central pathway for drug addiction and related behaviours in the brain (Picciotto and Corrigall, 2002). The α4β2 receptors present pre-synaptically on dopaminergic neurons of this pathway causing the release of dopamine and reinforcing the addictive properties of nicotine (Picciotto et al., 1998). Thus nicotinic receptors, particularly the α4β2 subtype, contribute to nicotine addiction through alterations of pre-synaptic release of dopamine in the brain.

Human epilepsy syndrome:

Mutations in genes encoding neuronal nAChRs have been determined to cause a form of sleep-related epilepsy called autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). The genes encoding for α4, α2 and β2 (CHRNA4, CHRNA2, and CHRNB2) subunits were implicated by ADNFLE (Marini and Guerrini, 2007). The α4 gene contains an S248F[2] mutation within the ion channel pore (Kuryatov et al., 1997). A change in the functional characteristics of the receptor due to gene mutations forms the basis of epileptogenesis (Mulley et al., 2003). In-vitro and in- vivo studies have shown that certain mutations in nAChRs present in the thalamus cause over activation of the brainstem's ascending cholinergic pathway and enhanced GABAergic neurotransmission in the reticular activating system which controls arousal from sleep and plays a central role in seizure precipitation in ADNFLE (Hogg et al., 2003).

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[2] Nomenclature for amino acid location and mutations within the primary sequence: Residues are designated by using the single letter amino acid code for the amino acid found in the "typical" or "normal" sequence along with its position in the primary sequence. Hence S248 refers to a serine at postion 248 numbered from the initiator methionine as position 1.A mutation would be designated as a second amino acid following the postion. For example S248F indicates that the serine 248 amino acids downstream from the initator methionine is mutated to phenylalanine. The species and subunit may also be included for clarity (ex: *h*α4S248F).

Autism:

Autism is a spectral disorder (Geurts et al., 2008) and in some autistic cases neuronal α4β2 receptor protein expression and receptor binding density as well as α4 mRNA levels in the parietal cortex is decreased (Lee et al., 2002). Reduced gene expression of the α4β2 receptors in the cerebral cortex is a major feature of the neuro-chemical pathology of Autism (Lee et al., 2002). The α7 receptors expression however remains unchanged (Lee et al., 2002) The precise role of nAChR expression changes in the etiology of Autism remains unclear; however, they may contribute to changes in "nicotinic tone" in affected brain regions. Nicotinic tone refers to the overall level of activation of synapses modulated by nicotine. High nicotinic tone would facilitate synaptic transmission in areas regulated by pre-synaptic nAChRs while low nicotinic tone would hinder transmission. Therapeutic agents that could restore or improve nicotinic transmission to compensate for decreased α4β2 receptor expression can play an important role in treatment options for Autism. This would represent an important advance in a disorder with no clear pharmacological intervention.

Schizophrenia:

Schizophrenia is a psychiatric disorder characterized by abnormal perceptions or expressions of reality manifested as auditory hallucinations, paranoid delusions or disorganized speech and thinking (Lake and Hurwitz, 2006). Sensory gating, an involuntary process by which selective attention is paid to sensory stimulus is profoundly impaired in schizophrenic patients (Leonard et al., 1996). A high level of nicotine addiction has been observed in schizophrenics and it is thought that smoking may restore normal sensory gating (Mobascher and Winterer, 2008). The temporal lobes of schizophrenics show a reduced α-bungarotoxin binding to nAChRs which are predominantly α7 receptors (Freedman et al., 1993). Sensory gating defects can be induced in mice by α-bungarotoxin administration which targets the α7 subtype of nAChRs (Luntz-Leybman et al., 1992). Many nAChRs

agonists have been shown to restore normal sensory gating in animal models of sensory gating deficits (Martin et al., 2004). However, partial agonists are found to function better than agonists in this regard; likely through decreased desensitization of α7 receptors (Stevens et al., 1998). Novel modulatory compounds targeting α7 receptors may provide new treatment options for Schizophrenia.

Alzheimer's disease (AD):

Characterized by dementia, AD affects 15 million people worldwide and this number is likely to grow as life expectancy increases (Palmer, 2002). A clear pathological feature of AD is the presence of amyloid plaques which arise from amyloid-β (Aβ 1-42) peptides and neurofibrillary tangles formed by the aggregates of hyperphosphorylated tau protein. Severe functional loss of cholinergic systems in the basal forebrains of patients suffering from AD has been observed (Perry, 1986). In advanced cases of AD cholinergic neurons are lost and the number of nAChRs reduced in the hippocampus and cortex (Coyle et al., 1983). Paradoxically, smokers have a lower prevalence of AD than non-smokers (Newhouse et al., 1988), presumably because nAChR expression levels are increased in the brains of smokers (Perry et al., 1999), possibly providing a neuro-protective mechanism against AD. The common treatment prescribed for AD is acetylcholinesterase inhibitors such as galantamine. In addition to being an acetylcholinesterase inhibitor galantamine also allosterically potentiates nAChRs present in the brain (Maelicke et al., 2001). Such drugs in AD patients are thought to restore normal functioning of cholinergic synapses through dual mechanisms of acetylcholinesterase inhibition and nAChR potentiation (Maelicke et al., 2001). Nicotinic agents like galantamine improve cognitive deficits of AD patients (Maelicke et al., 2001). The loss of pre-synaptic nAChRs due to Aβ1-42 toxicity causes improper release of other neurotransmitters such as glutamate in the hippocampus and this may also contribute to memory loss in AD patients. Loss of nAChRs in AD may cause mis-functioning of various

neuronal pathway activities and loss of synaptic plasticity. Drugs targeting specific subtypes of neuronal nicotinic receptors could be valuable therapeutic agents in AD since such drugs would restore cholinergic tone to the diseased brain.

1.11 Principles of Allostery:

Derived from the Greek word *allos* meaning "other" and *stereos* meaning "solid" or "shapes", allostery is the property of proteins to adopt a conformation or shape induced by the binding of two or more ligands. When two different ligands are involved in the allosteric interaction it is called heterotrophic allosterism (Lehninger et al., 2005). The allosteric effect is also seen when the same ligand binds to multiple similar sites on a single protein, known as homotrophic allosterism. The binding of Oxygen to haemoglobin is the most widely studied example of homotrophic allosterism in biochemistry

In multi-subunit proteins which bind two or more ligands at two distinct sites, the binding of one ligand generally increases or decreases the rate of binding of the second ligand to the protein (Taly et al., 2006). If the rate of binding of the second ligand increases due to the presence of the first ligand this effect is known as positive cooperativity; if binding decreases, the effect is known as negative cooperativity (Edelstein et al., 1997). The level of cooperativity is indicated by the Hill coefficient (Creighton, 1993) which gives a quantitative measure of the degree of cooperativity (Creighton, 1993). The Hill coefficient corresponds to the hypothetical number of ligand molecules that would have to be bound to the protein in an all-or-none fashion to display perfect cooperativity (Creighton, 1993). However the actual binding of a ligand to a protein is not totally cooperative, thus the Hill coefficient is seldom a perfect integer value. The value of the Hill coefficient can be as large as the number of interacting binding sites only if there was complete cooperativity between these sites (Creighton, 1993).

Jefferies Wyman, Jacques Monard, and Jean-Pierre Changeux in 1965 proposed an interesting theory to explain allosterism in proteins. This theory is known as the WMC Model or the Concerted Model of Allosteric Transition. According to the Concerted Model, proteins can exist in multiple inter-convertible conformations (Creighton, 1993). These conformations are determined by thermal equilibrium and this influences the conformational state of the protein at any given time. Binding of a ligand shifts this thermal equilibrium favourably for one conformation versus another (Creighton, 1993).

The Concerted Model assumes that multiple distinct conformations of a multi-subunit protein can exist (Creighton, 1993). A specific conformation has a higher affinity for the ligand than any other conformation the protein assumes (Creighton, 1993). While the ligand can bind the protein in any conformation, the conformational equilibrium is favoured for that conformation which has the highest affinity for the ligand. Thus, the change in concentration of the ligand over a small concentration range leads to a huge shift of equilibrium to the conformation favoured by the ligand. This explains the sigmoidal nature of the binding curves when ligands bind to multiple binding sites in allosteric multi-subunit protein (Hille, 2001).

Heterotrophic allosteric interactions involving other ligands occur because these ligands bind preferentially to one of the conformations that the protein assumes. Generally, each ligand controls the apparent affinity of the protein for other ligands by shifting the equilibrium towards the conformation that it prefers (Creighton, 1993).

1.11.1 Allosteric transition in nicotinic receptors:

Homotrophic allosteric transition in nAChR by acetylcholine binding to the orthosteric site: The concept of allosteric transition proposed by Wymann, Monod and Changeux was found to effectively explain the properties of nAChR (Karlin, 1967). This concept explains how the nAChR protein can exist in multiple distinct conformations. The closed state of the receptor (with no ion conductance) and the open state (ion conducting state) are the two

conformations identified (Del Castillo and Katz, 1957). The protein can exist in either conformation based on the thermal equilibrium. If the thermal equilibrium changes, the receptor can undergoes spontaneous conformational changes. The nAChR channels open with a low probability even in the absence of an agonist (Grosman and Auerbach, 2000). ACh, an agonist for the nAChR, binds and changes the conformational equilibrium to the open state of the receptor. If one ACh molecule could open the channel of the nAChR, than the conductance of a population of receptors with respect to ACh concentration would increase in sigmoidal fashion with a curve similar to the classic Michaelis-Menton equation for enzyme kinetics and the conductance would saturate at high ACh concentrations (Hille, 2001). The measured dose-response curve, however, showed approximately a four fold increase in conductance for every doubling of ACh concentration and a slope of almost two on a log plot (Lester et al., 1975). The dependence on the square of the agonist concentration implied that two agonist molecules were needed to open the channel. In a log-log plot of conductance versus concentration of ACh for the muscle nAChR, the Hill coefficient is 1.5-2.2 in most peer-reviewed literature (Hille, 2001). Biochemical studies confirmed that chemically purified muscle nACh receptors had two equivalent ACh binding sites formed by two α-subunits (Dunn et al., 1983).

Due to thermal fluctuations the nicotinic receptor channel can open spontaneously without the presence of an agonist (Colquhoun and Sakmann, 1981). If the channel can open spontaneously without an agonist and two ligands are needed to open it, even a single agonist bound state could open the channel. While the channel can open in any of the three scenarios, the presence of agonist increases the probability of channel gating. In the absence of an agonist, the channel opening probability is 1/1000000; with two bound agonists the probability increases to 1/33.

As per the concerted model of allosteric transition, the binding of the ligand shifts the equilibrium to a new conformation of the protein. Accordingly, the agonist for nAChR shifts

the equilibrium to the open state. The energy from the ligand binding interaction provides the energy required to stabilize this open conformation of the receptor (Lena and Changeux, 1993). Acetylcholine, an endogenous agonist for the nAChR, binds to a specific site on the receptor and stabilizes the open conformation of the channel. The binding site for ACh could be defined as the *orthosteric site* on the nicotinic receptor. The binding of two ACh molecules on the nicotinic receptor happens at two distinct identical sites (Hille, 2001) and is homotrophic allosteric in nature (Hille, 2001).

Heterotrophic allosteric transition in nicotinic receptor by ligand binding to allosteric site: The presence of multiple distinct binding sites on the nicotinic receptor for many ligands is possible. These sites would be different from the conventional ACh binding site and would form heterotrophic allosteric interactions. Ligands binding to such sites are *allosteric modulators* for the receptor (Bertrand and Gopalakrishnan, 2007). If an allosteric ligand further stabilizes the open confirmation of the receptor channel induced by the agonist, it is known as a *positive allosteric modulators* (PAMs) for the receptor (Bertrand and Gopalakrishnan, 2007). Positive allosteric modulators further shift the conformational equilibrium of the nicotinic receptor to the open state and increase the receptor response (Bertrand and Gopalakrishnan, 2007).

PAMs increase the receptor response in the presence of an agonist. However, it would be a misnomer to identify a ligand as a positive allosteric modulator if it can activate the channel by itself. A ligand can have an allosteric binding site on the receptor and still activate the channel on a microscopic or a macroscopic level. Such ligands may be classified as allosteric non-competitive agonists or allosteric partial agonists (Storch et al., 1995). A true positive allosteric modulator is one which binds to an allosteric site on the receptor, but fails to activate the channel by itself on a macroscopic or microscopic level. The presence of either a full or partial agonist is necessary for a positive allosteric modulator to open the

channel. A positive allosteric modulator must also show dose-dependent potentiation and inhibition of the receptor. It may increase efficacy, affinity or both when co-applied with an agonist or a partial agonist. A positive allosteric modulator can decrease the EC_{50} of the agonist which means increasing the apparent affinity of the agonist for the receptor. There are ligands known to act as *negative allosteric modulators* for nAChRs (Arias, 2000). Negative allosteric modulators shift the conformational equilibrium towards the closed state without directly competing with the agonist (Arias, 2000). These compounds increase the EC_{50} and move the dose-response curve for the agonist without directly competing for its binding site. Such compounds could potentially bind to the same allosteric site as a positive allosteric modulator on the receptor and competitively inhibit the actions of the positive allosteric modulator (Arias, 2000). For the benzodiazepine binding site on GABA (A) receptors, compounds such as Ro15-4513 and β-carbolines are known to act as negative allosteric modulators (Mehta and Ticku, 1999). Ro15-4513 and β-carbolines are negative allosteric modulators for the GABA (A) receptors.

1.12. Allosteric modulators for α7 and α4β2 subtype of nAChRs:

The neuronal nAChRs receptors are known to have multiple conformations where binding of a ligand preferentially stabilizing the receptor protein in a specific conformation (Edelstein et al., 1997). There are many ligands which are known to bind α7 and α4β2 subtypes of nAChRs on allosteric sites, separate from the orthosteric site where ACh binds (Bertrand and Gopalakrishnan, 2007). Such allosteric ligands for neuronal nAChR can produce a positive as well as a negative effect on the receptors' responses. Since the α7 and α4β2 are the two predominant subtypes of nAChR widely distributed in the brain, they are involved in the patho-physiology of many diseases and conditions, hence positive allosteric modulators for this receptor holds immense therapeutic potential (Gotti et al., 2006).

1.12.1. Allosteric modulation of α7 nAChRs:

The first compound identified as a positive allosteric modulator of the α7 receptor was an anthelminthic agent, ivermectin (Krause et al., 1998). This compound increased maximal ACh-evoked currents, reduced its EC_{50} value and increased the slope of the dose-response curve (Hill coefficient). A partial agonist for the α7 receptor, dimethylphenylpiperazinium (DMPP) became almost a full agonist when co-exposed with ivermectin (Krause et al., 1998). Genistein, a tyrosine kinase inhibitor was found to act as a positive allosteric modulator for α7 receptors that predominantly affected the peak current of the receptors (Charpantier et al., 2005). Later, several compounds which could increase the ACh induced currents at the α7 receptors were reported (Bertrand and Gopalakrishnan, 2007).

A novel positive allosteric modulator, PNU-120596 (1-(5-chloro-2, 4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea) potentiates α7 receptors but lacks action on α4β2, α3β4 and α9α10 receptors (Hurst et al., 2005). In a high-throughput screen, PNU-120596 increased agonist-evoked calcium flux mediated by human α7 receptors (Hajos et al., 2005). Electrophysiological studies with PNU-120596 on α7 receptors showed an increase in peak agonist-evoked currents and pronounced prolongation of the response in the presence of the agonist. With single channel studies PNU-120596 increased the channel mean open time of α7 receptors with no effect on ion selectivity (Hurst et al., 2005). Later studies identified the importance of the transmembrane regions in potentiation of the α7 receptors by PNU-120596 in presence of an agonist. Five transmembrane amino acid mutations significantly reduced potentiation of α7 receptors by this compound (Young et al., 2008). These amino acids are located in the α-helical transmembrane domains TM1 (S222 and A225), TM2 (M253) and TM4 (F455 and C459). Mutation of A225 or M253 individually showed a reduction in potentiation by PNU-120596 compared to wild type α7 receptors. In the homology models of the α7 receptor, based on the 4Å structure of the *Torpedo* nAChR, the side chains of these

five amino acids point toward an intra subunit cavity located between the four α-helical transmembrane domains; computer docking models suggest that PNU-120596 may bind within this intra-subunit cavity (Young et al., 2008). A number of other allosteric modulators of α7 receptors have been identified. 5-hydroxyindole (5-HI) produces a significant increase in the ACh-evoked current of α7 receptors (Zwart et al., 2002). SLURP-1, the secreted mammalian Ly-6/uPAR related protein (SLURP-1, a peptide secreted by human keratinocytes, is also known to be a positive allosteric modulator of human α7 receptors (Chimienti et al., 2003). SLURP-1 produces an increase in the ACh-evoked current on human α7 receptor, a reduction in the EC_{50} for ACh and an increase in the slope of the concentration response curve (Chimienti et al., 2003).

1.12.2. Non-selective positive allosteric modulators of α4β2 receptors:

Due to their wide distribution, α4β2 receptors are involved in the patho-physiology of many neurological conditions. Schizophrenia, Autism, loss of cognitive function and nicotine addiction are some of the diseases and conditions where α4β2 receptors are thought to be involved (Gotti and Clementi, 2004). Positive allosteric modulators are potentially useful as therapeutic agents since they may be capable of restoring or improving nicotinic tone as a result of decreased α4β2 receptor expression.

Galantamine and Physostigmine: The acetylcholinesterase inhibitor physostigmine has been shown to directly activate muscle type nAChRs in frog muscle fibers and *Torpedo* nAChR (Shaw et al., 1985). This effect of physostigmine is distinct from its inhibitory action on the acetylcholinesterase enzyme (Albuquerque et al., 1988). The agonist action of physostigmine is inhibited by the nAChR specific monoclonal antibody FK1, but not by nAChR competitive ligands (Okonjo et al., 1991). The epitope for this monoclonal antibody is located between amino acids 118 and 145 on the α-subunit of the nAChR. Photo–affinity labelling of *Torpedo*

nAChR with [^{3}H]-physostigmine has shown that physostigmine binds to the α-subunit of the receptor near K125 (Schrattenholz et al., 1993). Photo-affinity labelling and inhibition studies with FK1 confirm that physostigmine and the FK1 antibody share overlapping binding sites. In a similar study, the binding site for acetylcholine on the α-subunit of the nAChR was found to overlap the epitope for the monoclonal antibody WF6. The WF6 epitope is located around amino acids 81 to 200 overlapping the ACh orthosteric binding site on α1 (Conti-Tronconi et al., 1991). Taken together, these studies show that acetylcholine and physostigmine bind at different binding sites on the α1 receptor making physostigmine a unique non-competitive allosteric agonist (Pereira et al., 1993). Galantamine, another cholinesterase inhibitor and the opiod codeine have also been confirmed to be non-competitive nAChR allosteric agonists. The binding site for these compounds is thought to be similar to that of physostigmine (Storch et al., 1995).

Physostigmine and galantamine (Figure 1.10) show effects on α4β2 receptors that are similar to their action on muscle receptors and are thought to activate the receptor via a different binding site than ACh (Pereira et al., 1994). Physostigmine activates chicken α4β2 receptors expressed in M10 cells, through a binding sight that is also insensitive to ACh (Pereira et al., 1994), but did not elicit currents on the same receptor when expressed in *Xenopus* oocytes. When physostigmine is co-applied with acetylcholine on α4β2 receptors expressed in *Xenopus* oocytes, it potentiates ionic currents induced by low concentrations of acetylcholine with inhibition at higher acetylcholine concentrations (Smulders et al., 2005). Physostigmine has been shown to bind to a low affinity ACh binding site present on α4β2 receptors distinct from the high affinity site that activates the receptor. Physostigmine potentiation of α4β2 receptors is only seen in the presence of low concentrations of acetylcholine (Smulders et al., 2005).

Galantamine has been used clinically to combat the symptoms in Alzheimer's disease (Scott and Goa, 2000). Its clinical use is based on both its ability to inhibit

acetylcholinesterase in the synapse and to simultaneously potentiate both α7 and α4β2 receptors (Maelicke et al., 2000). In HEK293 cells expressing human α4β2 receptors, galantamine reduces the apparent EC_{50} for ACh by half but has no effect on the efficacy as measured by the apparent I_{max} (Samochocki et al., 2003). In addition to α4β2 and α7 receptors, galantamine also potentiates α3β4 and α6β4 (Maelicke et al., 2000). The proposition that galantamine binds to an allosteric site on nAChR is strongly supported by experiments and modeling studies which reveal that this compound binds to the *Aplysia Californica* AChBP in a region different from the ACh binding site (Hansen and Taylor, 2007).

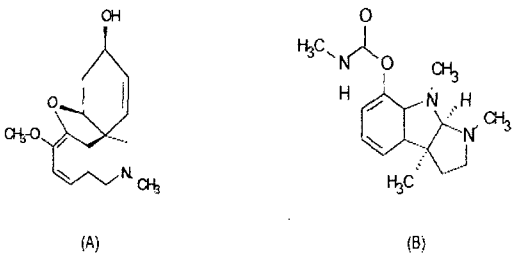


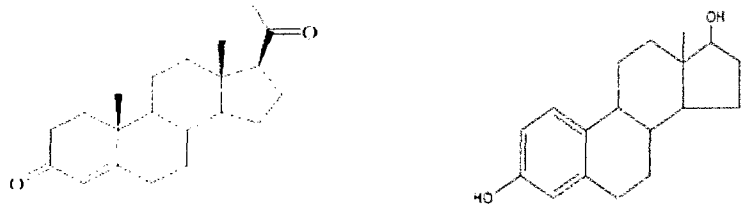
Figure 1.9: Structure of (A) Galantamine and (B) Physostigmine

Physostigmine and galantamine are both thought to potentiate neuronal nAChRs by stabilizing the open state of the receptor channel (Maelicke et al., 2001). Galantamine and physostigmine are non-selective in that they are known to potentiate both homomeric α7 receptors and the heteromeric subtypes of nAChRs. The beneficial action of these two drugs in restoring cholinergic tone in the synapse is expected to be enhanced by their dual actions as acetylcholinesterase inhibitors and allosteric modulators of nAChRs (Maelicke et al., 2000).

Steroids: The primary mechanistic action of steroid hormones derived from cholesterol is to control gene transcription through activation of intracellular receptors. This is a slow process

since steroid signalling involves uptake of the steroid into the cell via an endocytotic process followed by binding to appropriate regulatory sites for gene transcription and translation. However, certain neuro-steroids are also capable of modifying neuronal activity through mechanisms that are too fast to involve gene expression. Neuro-steroids are synthesized in glial and neuronal cells in the brain and act through the steroid hormone nuclear receptors, and other mechanisms including modulation of neurotransmitter gated ion channels (Mellon et al., 2001). Steroid injections have been shown to improve cognition, memory and learning (Frye and Sturgis, 1995). The first evidence of a steroid targeting α4β2 receptors was provided by an experiment in which water soluble serum albumin-attached progesterone and unbound progesterone effects on α4-containing receptors were compared. This experiment showed that albumin-bound and unbound progesterone are equally capable of inhibiting α4-containing receptor activity in *Xenopus* oocytes. Since albumin-bound progesterone can not cross the cell membrane, this supported an extracellular site of action for progesterone on the α4 subunit of the nAChRs (Valera et al., 1992). Progesterone was tested for its ability to potentiate α4β2 receptors, but it failed to do so. However, 17-β-Estradiol (17-BE) potentiated human α4β2 receptors and inhibited rat α4β2 receptors without potentiation (Paradiso et al., 2000). The difference in action of 17-BE in the two species was used to locate the binding site on the α4β2 receptor responsible for potentiation. Using site directed mutagenesis, it was determined that the short C-terminal end of the α4 subunit with the amino acid sequence WLAGMI was the key determinant for 17-BE potentiation of human α4β2 receptors. The tryptophan (W) in this C-terminal sequence has been hypothesized to form a specific π-π interaction with the ethynyl moiety of 17-BE. The binding site of 17-BE for potentiation was shown to be different than the steroid inhibition site because the mutation in the C-terminal sequence has no effect on progesterone inhibition (Paradiso et al., 2001). The complex modulation of α4β2 receptors by steroids seems to be determined by both the chemical structure of the steroid and the amino acid sequence of the nAChR subtypes. There is

growing evidence to suggest a transmembrane location of an inhibitory binding site for different steroids in other subtypes of nAChRs (Arias, 1998). Experiments attempting to locate such a binding site have not been carried out in α4β2 receptors. Single channel studies have confirmed that mechanistically, 17-BE increases the opening probability of α4β2 receptors (Curtis et al., 2002).



(A) (B)

Figure 1.10: Structure of (A) Progesterone; (B) 17-β-Estradiol

Zinc: Ionic zinc (Zn^{2+}) is a key modulator of neuronal excitability which is present in neurons throughout the brain, specially in the cerebral cortex and the hippocampus (Frederickson et al., 2000). Zinc plays an important role in modulating synaptic activity (Howell et al., 1984) and modulates the function of many LGIC receptors including GABA type A (Krishek et al., 1998) and glycine receptors (Harvey et al., 1999). Zinc was found to inhibit α7 (Palma et al., 1998) and α3β2 receptors. When tested for its action on neuronal nAChR, it produced a biphasic dose response on α2β2, α2β4, α3β4, α4β2 and α4β4 subtypes (Hsiao et al., 2001). ACh generated currents on α2β2, α2β4, α3β4, α4β2 and α4β4 subtypes of nAChRs were potentiated by zinc at low μM concentrations and inhibited at a higher mM concentration range. For the α4β2 receptor, the half-maximal concentration for potentiation (EC_{50}) was about 16 μM and the half-maximal concentration for inhibition (IC_{50}) was about 440 μM (Hsiao et al., 2001). ACh responses on α4β2 receptors were potentiated by 260% with zinc.

Application of 50 μM zinc increased the response of α4β2 receptors at saturating ACh concentrations (Hsiao et al., 2001). Zinc potentiation and inhibition are both pH dependent, but voltage independent (Moroni et al., 2008). By site directed mutagenesis and the substituted cysteine accessibility method (SCAM) certain key residues of the β2+/α4- interface of α4β2 receptor have been identified as critical to zinc potentiation (Hsiao et al., 2006). Of the two different stoichiometries of the α4β2 receptor in the central nervous system, zinc potentiates only the low ACh affinity stoichiometry $(\alpha4)_3(\beta2)_2$ receptors while inhibiting both high and low affinity stoichiometry of the receptor (Moroni et al., 2008). The inhibition of Zinc on the high affinity $(\alpha4)_2(\beta2)_3$ receptor is voltage dependent while it is voltage independent on low affinity $(\alpha4)_3(\beta2)_2$ receptors (Moroni et al., 2008). Based on these observations and through site directed mutagenesis studies, the $\beta2^+/\alpha4^-$ subunit interfaces were proposed as the Zn^{2+} inhibition site on $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ receptors and the $\alpha4^+/\alpha4^-$ subunit interface was proposed as the site for the potentiating action of Zn^{2+} on $(\alpha4)_3(\beta2)_2$ receptors (Moroni et al., 2008). Single channel studies suggest Zn^{2+} potentiates α4β4 receptors by increasing the burst frequency of the receptor (Hsiao et al., 2008). Since Zn^{2+} potentiates α4β4 and α4β2 receptors, the mechanism of action of zinc on α4β2 receptors may be similar to its mechanism of action on α4β4 receptors.

Thiazole(2-amino-5-keto) and Carbamate Analogues: Three novel (2-amino-5-keto) thiazole analogues have been reported allosteric modulators of neuronal nAChRs (Broad et al., 2006). These compounds potentiated α2β4, α4β2, α4β4, α7 but not α3β2 and α3β4 receptors (Broad et al., 2006). Unlike the previously discussed α4β2 potentiators galantamine and physostigmine, none of these compounds are acetylcholinesterase inhibitors (Broad et al., 2006). All three compounds enhanced the potency and maximal efficacy of different nicotinic agonists on α4β2 receptors, a profile typical of allosteric potentiators. At concentrations required for potentiation, the compounds did not displace

[^{3}H]-epibatidine from the agonist-binding site, and potentiation was observed at all agonist concentrations, suggesting a non-competitive allosteric mechanism of action. Interestingly, at concentrations higher than that required for potentiation these compounds also showed intrinsic agonist activity which was blocked by competitive and non-competitive nAChR antagonists (Broad et al., 2006).

Very recently a series of carbamate analogues were reported as α4β2 nAChR potentiators. In addition to α4β2 receptors these molecules also bound to α3β2 and α3β4 receptors and were optimized for CNS penetration (Albrecht et al., 2008). These compounds also increased the response of α4β2 receptors to ACh (Springer et al., 2008) without competing for binding with the orthosteric ligand cytosine (Albrecht et al., 2008). These carbamate analogues represent a new class of compounds with immense therapeutic potential, possibly acting by an allosteric site on α4β2 receptors. It has not been reported if these carbamate analogues do or do not have agonist activity.

Desformylflustrabromine (dFBr): Many alkaloid compounds from natural sources are known to act as agonists or antagonists for nAChRs. Epibatidine found in the skin of a neo-tropical poisonous frog, *Epipedobates tricolor* is a potent agonist of nAChRs (Daly, 2005). Curare extracted from *Chondrodendron tomentosum*, a plant found in South American jungles, contains an active component *d*-tubocurarine, which is an antagonist for nAChRs (Bowman, 2006). Desformylflustrabromine (dFBr) is a metabolite of the marine bryozoan *Flustra foliacea* which is common in the North Sea (Lysek et al., 2002). The structure of dFBr is derived from a brominated indol ring as seen in Figure 1.12.

Some of the tryptophan derived metabolites of *Flustra foliacea* were first shown to have muscle relaxant properties (Sjoblom et al., 1983). Flustramine A, another Flustra extract could also block potassium channels (Peters et al., 2002). When the Flustra derivatives were tested on α4β2 and α7 subtypes of the neuronal nAChR using radioligand

binding assays, it was determined that dFBr had low μM affinities for the nAChRs (Peters et al., 2004). dFBr extracted from its natural source was tested functionally on heteromeric α4β4, α3β2, α3β4, α4β2 and α7 nAChR expressed in *Xenopus* oocytes (Sala et al., 2005). When co-applied with ACh, dFBr selectively potentiated but did not inhibit α4β2 receptors. At the applied concentration, dFBr had no potentiating effect on other heteromeric α4β4 or α3β2 receptor but showed an inhibitory effect on homomeric α7 receptors. The potentiation induced by dFBr on ACh induced currents was reversible and concentration dependent. In single channel experiments, no changes in conductance or reversal of potential were seen. When the single channel data was subjected to dwell time analysis, it indicated that dFBr acts by either increasing the opening rate constant or decreasing the closing rate constant of α4β2 receptors (Sala et al., 2005).

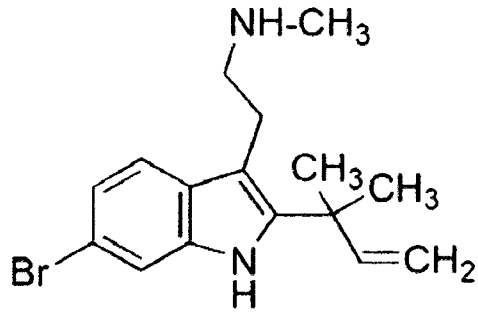


Figure 1.11: Structure of desformylflustrabromine (dFBr)

Drugs of the cholinergic system, 5-HT & codeine: Cholinergic drugs like atropine, scopolamine, rivastigmine, and tacrine have also been tested for their ability to potentiate α4β2 receptors (Smulders et al., 2005). Physostigmine-like compounds such as atropine, scopolamine and tacrine combine potentiation of an ion current induced by low concentrations of acetylcholine with inhibition of ion currents evoked by high concentrations of acetylcholine. Only rivastigmine causes inhibition of ion currents evoked by low concentrations of acetylcholine. No potentiation is observed. (Smulders et al., 2005).

Codeine, serotonin and the muscle relaxant benzoquinonium have been described as non-competitive allosteric agonists for nAChR, but have different actions on α4β2 receptors. The endogenous neurotransmitter 5-HT has also been shown to potentiate agonist activated currents for nicotinic receptors in PC12 cells (Schrattenholz et al., 1996). When expressed in *Xenopus* oocytes however, α4β2 receptors are inhibited by 5-HT in a non-competitive manner by a channel blocking mechanism (Garcia-Colunga and Miledi, 1995), but no potentiation is observed. Codeine, an opiate, potentiates nicotinic receptors by binding to the FK1 antibody site (Storch et al., 1995), though such action has not been demonstrated on α4β2 receptors.

Since nAChRs are implicated in the patho-physiology of many diseases and conditions, positive allosteric modulators for these receptors could be important therapeutic agents in treatment of neuro-pathological conditions where these receptors are implicated. The benzodiazepine class of compound, for example, is an amazing success story in the related GABA receptor (Mehta and Ticku, 1999). Allosteric modulatory ligands for nAChRs will lead to clinically applicable drugs targeting these receptors. Such compounds will provide a novel means of treatment for various neurological disorders where these receptors play a critical role.

1.13 Research Hypothesis:

1.13.1 Basic premise of studying allosteric modulators for nAChRs:

The principle reasons for studying allosteric modulation in nAChRs are:

1) To identify new mechanism of receptor function. Conventional agonists, antagonists and partial agonist act on the nicotinic receptors via interaction with the orthosteric site. Study of allosteric modulation may reveal new mechanisms of receptor activation.

2) To identify novel binding sites on neuronal nicotinic receptors for unconventional ligands. Neuronal nAChRs may contain multiple distinct sites for the binding of different ligands.

Using novel ligands as molecular probes, putative allosteric sites on nicotinic receptors can be found and studied. Experimental methods like Site directed mutagenesis, chimeric constructs and the substituted cysteine accessibility method (SCAM) can be utilized to pinpoint the amino acid residues in the allosteric binding site and their involvement in binding of allosteric ligands.

3) To discover novel allosteric modulatory ligands for neuronal nAChRs as lead molecules and develop them into clinically applicable drugs. One purpose of basic pharmacological research is to produce novel drug therapies. The discovery of novel allosteric modulators could provide new drug compounds which selectively target nAChR subtypes and lead to new treatment options for diseases and conditions where nicotinic receptors are involved. Since the large majority of nAChR modulators are non-selective, the development of subtype selective ligands will facilitate identification of the role of select subtype in disease processes.

1.13.2 General hypothesis:

Members of the ligand gated ion channel super-family (LGICS) include 5-HT_3R, nAChRs, GABA (A) and (C) receptors, glycine receptors and the acetylcholine binding protein (AChBP). All the members of this family are allosteric proteins (Edelstein et al., 1997). There are many available drugs such as benzodiazepines and barbiturates that act as alosteric modulators for GABA (A). These drugs are prescribed clinically as anti-depressants, anxiolytics and anaesthetics (Mehta and Ticku, 1999). However, there are no clinically available drugs that target neuronal nAChRs with selectivity for a specific subtype of nicotinic receptor. Based on preliminary molecular modelling studies and sequence comparison between members of the LGIC family, we formed the general hypothesis that **dFBr is a subtype selective positive allosteric modulator of neuronal nAChRs.** As such, dFBr will serve as a lead molecule for the development of a novel drug class with applicable to enhancing nicotinic synaptic transmission.

1.13.3 Specific hypotheses:

1) Synthetic dFBr is a selective[3] positive allosteric modulator of the α4β2 subtype of the neuronal nAChR with no potentiating activity on the α7 subtype of neuronal nicotinic receptors.

This hypothesis is based on previous studies that used dFBr from its natural source, the marine bryozoan *Flustra foliacea* and examined its effects on both α4β2 and α7 subtypes of nAChRs. We used a synthetic water-soluble HCl salt of dFBr in our studies. The experiments evaluating the effects of synthetic dFBr on α4β2 and α7 subtype nAChRs are described in Chapter 2 of this thesis. These experiments were designed to determine if the synthetic compounds exhibited similar effects to the naturally extracted dFBr.

2) Different functional groups in the chemical structure of dFBr are responsible for the potentiation versus inhibition of acetylcholine induced responses on α4β2 receptors.

While structurally similar, the chemical analogues of dFBr presented in this thesis contain different functional groups. These differences in structure are responsible for the differences in potentiation and inhibition observed on α4β2 receptors. In other words, we hypothesize that inhibition and potentiation occur due to binding of dFBr on different binding sites present on α4β2 receptors, each with unique structural requirements. Different dFBr analogues will produce different effects on potentiation and inhibition of ACh induced responses on α4β2 receptors. Structural modification of the parent compound dFBr would be expected to produce different effects on the potentiation and inhibition properties observed for dFBr. Based on this hypothesis, 16 analogues of dFBr were tested on α4β2 and α7 receptors. The results of these experiments are discussed in Chapter 3 of this thesis. These

[3] The term "selective" within the context of this thesis refers only to a relatively narrow definition of selectivity within the acetylcholine receptor family. This does not imply that the compound does not bind to other LGIC or non-LGIC receptors. Further testing beyond the scope of this thesis is required to validate claims of broad rather then narrow definitions of selectivity.

experiments were not designed to determine broad subtype selectivity of dFBr other than a simple comparison of α4β2 to α7 receptors, however, these data form the basis by which subtype selectivity can be examined in future studies in the laboratory.

3) The effects of dFBr are due to its binding at a unique binding site that is different from the binding site for other α4β2 receptor allosteric modulators.

Since the action of dFBr is selective for the α4β2 receptor subtype while physostigmine, Zn^{2+} and 17-β estradiol non-selectively potentiate other nicotinic receptor subtypes, we hypothesize that the binding site for dFBr will only be present on the α4β2 receptor. Physostigmine, Zn^{2+} and 17-β estradiol which also potentiate α4β2 receptors would not be expected to bind to this presumed dFBr binding site. This hypothesis will be tested pharmacologically by first evaluating the effects of all dFBr, physostigmine, Zn^{2+} and 17- β estradiol on a common system (a4b2 receptors expressed in Xenopus oocytes) than through combination studies in which two potentiators are co-applied. The results of these experiments are presented and discussed in Chapter 4 of this thesis.

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CHAPTER 2: ACTION OF SYNTHETIC DESFORMYLFLUSTRABROMINE ON ALPHA7 AND ALPHA4 BETA2 SUBTYPE OF NEURONAL NICOTINIC RECEPTORS[1]

2.1 Abstract:

Desformylflustrabromine (dFBr; **1**) and desformylflustrabromine B (dFBr-B; **2**) have been previously isolated from natural sources, and the former has been demonstrated to be a novel and selective positive allosteric modulator of $\alpha4\beta2$ nicotinic acetylcholine receptors (nAChR). The present study describes the synthesis of water-soluble salts of **1** and **2**, and confirms and further investigates the actions **1** and **2** using two-electrode voltage clamp recordings. When obtained by extraction from the natural source, dFBr potentiated ACh induced responses of $\alpha4\beta2$ receptors and appeared selective for the $\alpha4\beta2$ nAChR subtype compared to other common nicotinic receptor subtypes including the $\alpha7$ receptor. The synthetic HCl salts described here showed enhanced solubility and potency compared to the naturally obtained compound. In addition, a second inhibitory effect was observed at higher dFBr concentrations for $\alpha4\beta2$ and $\alpha7$ receptors. No potentiation of responses was observed on $\alpha7$ receptors. While dFBr is a positive allosteric modulators of $\alpha4\beta2$ receptor it lacks such an action on $\alpha7$ receptors. dFBr-B is not a positive allosteric modulator for $\alpha4\beta2$ or $\alpha7$ nicotinic acetylcholine receptors but is capable of inhibiting both receptor subtypes.

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[1] This data presented in this chapter has been previously published as a peer-reviewed research article: J.-S. Kim, A. Pandya, M. Weltzin, B. W. Edmonds, M. K. Schulte, and R. A. Glennon. (2007). "Synthesis of desformylflustrabromine and its evaluation as an alpha4beta2 and alpha7 nACh receptor modulator." Bioorganic Medicinal Chemistry Letters **17**(17): 4855-60.
The experimental data presented in this chapter is entirely my work, except for a portion of the data in Figure 2.3. Two repeats of this experiment were performed by Maegan Weltzin and two repeats were performed by me. The compounds tested were synthesized by J.-S. Kim and R. A. Glennon at the Virginia Commonwealth University, School of Pharmacy, Department of Medicinal Chemistry, Richmond, VA. The synthesis of the compounds is not included in this chapter.

2.2 Introduction:

Over the past decade or so, nicotinic acetylcholine receptors (nAChRs) have been targeted for the development of agents with potential for the treatment of certain cardiovascular and neurological (e.g. Parkinson's disease, Schizophrenia, Alzheimer's disease) disorders, memory and learning deficits, appetite control problems, and pain (Arias, 2000; Glennon, 2004). These pentameric ion channel receptors are composed of heteromeric or homomeric assemblies and, although numerous subtypes have been identified, the most prevalent in the brain are the heteromeric $\alpha4\beta2$ and the homomeric $\alpha7$ nAChRs (Arias, 2000; Glennon, 2004). While some recently developed ligands differentiate between the $\alpha4\beta2$ and $\alpha7$ sub-type of nAChRs, only a few (if any) have been shown to selectively target either $\alpha4\beta2$ or any other nACh receptor subtypes (Glennon, 2004). A novel strategy for modulation of nACh receptors is the identification of allosteric modulators that interact with receptors at a site distinct from that of the endogenous ligand. These agents modulate the receptor protein via a non-competitive mechanism. Negative allosteric modulators of nACh receptors have received some attention (reviewed in (Arias, 1998; Arias, 2000; Arias et al., 2006). Some compounds, specially the acetylcholinesterase inhibitors are known to be positive nAChR allosteric modulators (or non-competitive agonists) (Albuquerque et al., 2001; Arias, 2000; Hurst et al., 2005; Pereira et al., 2002). However, it is not yet known precisely how they work (Samochocki et al., 2003; Smulders et al., 2005). Nevertheless, several distinct binding domains of the latter agents already have been proposed from docking studies using models of $\alpha3\beta4$, $\alpha4\beta2$, and $\alpha7$ nACh receptors (Arias, 2000; Iorga et al., 2006)

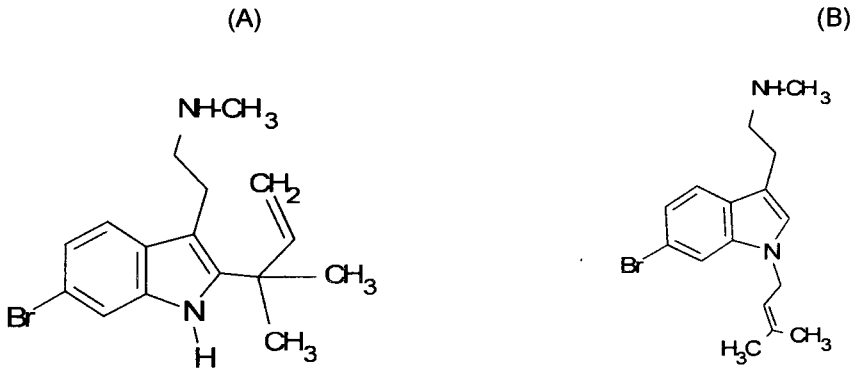

Figure 2.1: (A) Structure of desformylflustrabromine (B) Structure of desformylflustrabromine-B

Desformylflustrabromine (Figure 2.1.A) was identified as a positive allosteric modulator of nACh receptors (Sala et al., 2005). Desformylflustrabromine (dFBr) and desformylflustrabromine-B (Figure 2.1.B), and at least a dozen additional indolic alkaloids have been isolated and characterized from the marine bryozoan *Flustra foliacea* (Lysek et al., 2002; Peters et al., 2002; Peters et al., 2004). In particular, dFBr and dFBr-B lack appreciable affinity for $\alpha4\beta2$ receptors, and bind with >3,500-fold lower affinity at this receptor subtype than the standard agonist (–)-nicotine (K_i <1 nM); dFBr displays even lower affinity (K_i >50,000 nM) for $\alpha7$ nACh receptors (Peters et al., 2004). However, it has been further demonstrated with several heteromeric (i.e., $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$ and $\alpha4\beta4$) and the homomeric $\alpha7$ nACh receptors expressed in *Xenopus* oocytes, that dFBr, but not dFBr-B, selectively increases the whole cell current obtained when co-applied with the endogenous agonist acetylcholine in $\alpha4\beta2$-containing preparations (Sala et al., 2005). Single channel studies with dFBr are consistent with the hypothesis that it increases the channel open probability, perhaps by increasing the ratio of the rate constants for channel opening and closing (Sala et al., 2005). As such, dFBr offers a novel tool for the investigation of nACh receptors, and serves as a lead structure for the development of selective agents with

potential therapeutic application. To date, studies with dFBr and dFBr-B have employed dimethyl sulfoxide (DMSO) solutions of their water-insoluble free bases as isolated directly from the marine organism. The purpose of this study was to confirm the actions of synthetic dFBr and dFBr-B as selective allosteric potentiators of $\alpha4\beta2$ receptors and the lack of such action on $\alpha7$ receptors. We evaluated the actions of synthetic dFBr and dFBr-B as their HCl salts at concentrations ranging from 1 nM to 100 μM on human $\alpha7$ and $\alpha4\beta2$ nACh receptors expressed in *Xenopus* oocytes with two-electrode voltage clamp techniques.

2.3 Materials and Methods:

The chimeric DNAs (cDNA) for human $\alpha7$ and $\alpha4\beta2$ receptors were obtained from Dr. Jon Lindstrom's laboratory (Department of Neuroscience, School of Medicine, University of Pennsylvania). Subunit of cDNA was cloned into a pBud-CE4.1 (Invitrogen, CA) vector prior to mRNA synthesis. *Xenopus laevis* frogs and frog food were purchased from Xenopus Express (Homosassa, FL). Ovarian lobes were surgically removed from *Xenopus laevis* frogs and washed twice in Ca^{+2}-free Barth's buffer (82.5 mM NaCl / 2.5 mM KCl / 1mM $MgCl_2$ / 5 mM HEPES, pH 7.4) then gently shaken with 1.5 mg/ml collagenase (Sigma type II, Sigma-Aldrich) for 1 h at 20-25 °C. Stage IV oocytes were selected for microinjection. Synthetic cRNA transcripts for human $\alpha7$ and $\alpha4\beta2$ were prepared using the mMESSAGE mMACHINE™ High Yield Capped RNA Transcription Kit (Ambion, TX). Oocytes were injected with a total of 50 nL cRNA at a concentration of 0.2 ng/nL in appropriate subunit ratios then incubated at 19 °C for 24 to 72 h prior to their use in voltage clamp experiments. Recordings were made using an automated two-electrode voltage-clamp system incorporating an OC-725C oocyte clamp amplifier (Warner Instruments, CT) coupled to a computerized data acquisition (Datapac 2000, RUN technologies) and autoinjection system (Gilson). Recording and current electrodes with resistance of 1-4 MΩ were filled with 3 molar

KCl. Details of the chambers and methodology employed for electrophysiological recordings have been described earlier (Joshi et al., 2004). Oocytes were held in a vertical flow chamber of 200-μL volume and perfused with ND-96 recording buffer (96 mM NaCl / 2 mM KCl /1.8 mM $CaCl_2$ / 1 mM $MgCl_2$ / 5 mM HEPES; pH 7.4) at a rate of 20 ml/min. Test compounds were dissolved in ND-96 buffer and injected into the chamber at a rate of 20 ml/min using the Gilson auto-sampler injection system. For dFBr and dFBr-B experiments, compounds were co-applied with 100 μM ACh.

2.3.1 Data Analysis:

Concentration-response curves were fit by non-linear curve fitting and GraphPad Prism Software (San Diego, CA) using standard built-in algorithms. For the potentiation/inhibition curves obtained for α4β2 modulation by dFBr·HCl the data was fit to a bell shaped dose-response equation based on a hormetic model. The hormetic model simultaneously fits both the potentiation and inhibition phases of the response to give an EC_{50} for the potentiation component and an IC_{50} for the inhibitory component. Mathematically, this is equivalent to fitting a stimulatory and inhibitory equation simultaneously. Data was fit to the following equation:

$$I = (1 + ((Im - 1)/(1 + 10^{((logEC50-X)*nHp)}))) / (1 + 10^{((logIC50))*nHi)}) \quad \textbf{2.1}$$

Where, I = fractional current obtained at a given ligand concentration, Im = I_{max} (maximum theoretical current obtainable by the potentiator), EC_{50} = concentration of potentiators producing I = ½ I_{max}, IC_{50} = concentration of potentiators inhibiting to ½ I_{max}, nHp = Hill slope for potentiation, nHi = Hill slope for inhibition.

The accuracy by which this algorithm separates these two constants is dependant on the degree of difference between them. In the case of dFBr·HCl and dFBr-B·HCl, this

difference is about 33-fold. For IC_{50} determinations data were fit to a single site competition model, equation:

$$y = 1/[1 + (IC_{50}/[antagonist])^{nH}] \quad \textbf{2.2}$$

Where, nH is the Hill coefficient.

Statistic analysis: Quantitative data are expressed as mean ± S.E. Associations between the variables, where needed, were tested by using paired stunents-*t* test. All statistical differences were deemed significant at the level of $P < 0.05$.

2.4 Results:

2.4.1 Action of dFBr on α7 receptors

Application of 100 μM ACh produced a near maximal response in α7 receptors (Figure. 2.2). Co-application of >0.1 μM dFBr and 100 μM ACh inhibited the response to ACh. No potentiation of α7 responses was observed over the range of dFBr concentrations tested. A dose response curve obtained from multiple experiments identical to that shown in Figure 2.2A is plotted in Figure 2.2B. Peak amplitudes of currents obtained from α7 receptors were inhibited by dFBr (IC_{50} = 44.1 ± 1.25 μM). The low potency for dFBr inhibition is consistent with its low affinity for the receptors (Peters et al., 2002). Our data demonstrate an inhibitory action of dFBr on α7 receptors. Sala et al (Sala et al., 2005) also observed slight inhibition of α7 receptors by dFBr isolated from *Flustra foliacea*.

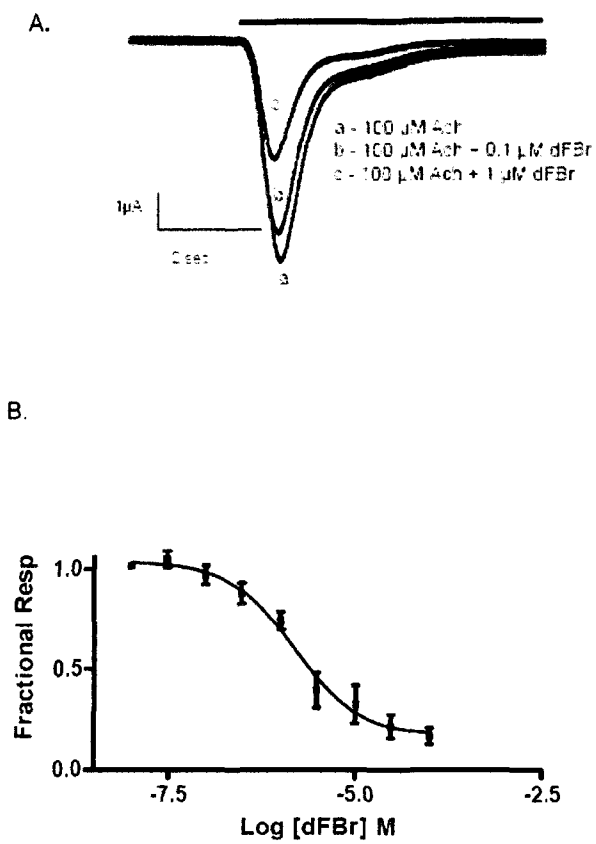

Figure 2.2: Effects of dFBr on α7 nACh receptors dFBr (1 nM to 100 μM) was co-applied with 100 μM ACh to Xenopus oocytes expressing human α7 nACh receptors. Responses were obtained using two-electrode voltage clamp at a -60 mV holding potential. Drug exposure is indicated by the horizontal bar above the traces. Peak amplitudes were normalized to the responses obtained using 100 μM ACh in the absence of dFBr (1). A: Currents elicited by 100 μM ACh at dFBr 1 concentrations of 0μM, 0.1μM and 1.0μM. B: Concentration-response curve for dFBr inhibition of peak currents elicited by 100 μM ACh (IC_{50}=44.1 +/- 1.25 μM).

2.4.2 Action of dFBr on $\alpha 4\beta 2$ receptors

On $\alpha 4\beta 2$ receptors, co-application of 100 μM ACh and dFBr produced a biphasic response over the concentration range tested (Figure 2.3). Responses elicited by 100 μM ACh were potentiated by dFBr (0.001 μM to 100 μM), although inhibition of the response was observed at concentrations greater than 10 μM dFBr, producing a bell shaped dose-response curve. Similar results were obtained when the slope of the rise time of the response was plotted rather than peak amplitude (data not shown). The IC_{50} value estimated for the inhibitory component of this curve is 6.01 ± 0.92 μM. This value is about twice than that reported by Peters et al (Peters et al., 2002) 3.4 μM for inhibition of ^{3}H-epibatidine binding on rat $\alpha 4\beta 2$ receptors. Similar to our data, Sala et al. (Sala et al., 2005) did not report inhibition of ACh responses at these concentrations of dFBr but observed potentiation at 3 μM. While Peters' data showed displacement of ^{3}H-epibatidine binding it is not clear if this is due to competitive or non-competitive effects. If dFBr binding to an allosteric potentiation site reduces the affinity of the antagonist epibatidine, than dFBr could appear as an inhibitor in a binding assay (Peters et al., 20002) and as a potentiator of acetylcholine responses in a functional assay (Sala et al., 2005) Unlike Sala, we did observe inhibition of 100 μM ACh responses at high dFBr concentrations (> 10 μM). However, the current traces obtained at these concentrations were not typical of those resulting from displacement of agonist. Displacement of an agonist typically produces a decrease in overall response amplitude with little change in response profiles. In our study, high dFBr concentrations produced currents with a greater increase in the apparent rate and extent of desensitization rather than a simple decrease in overall response amplitudes (see Figure 2.2A). This is particularly evident when comparing equal amplitude responses in the presence of dFBr (Figure 2.3 A c and d); trace c (1 μM dFBr – a sub-inhibiting dFBr concentration) shows a relatively slow apparent rate of desensitization while trace d (10 μM – an inhibiting dFBr concentration) shows a much

sharper peak with a faster apparent rate of desensitization. If inhibition were due to displacement of ACh binding, these two response profiles would be expected to be similar. We also typically observed rebound currents on washout of Ach-dFBr (Figure 2.4). These currents are commonly observed with open-channel block due to removal of the channel blocker prior to dissociation of the agonist from the receptor. The rebound currents are particularly evident in the 10 μM responses as shown in Figure 2.4. Our data support the hypothesis that open channel block contributes to inhibition of ACh responses by dFBr. Similar effects have been observed for other potentiating ligands including physostigmine and levamisole (Albuquerque et al., 2001; Levandoski et al., 2003). Thus, the inhibition we observe at high dFBr concentrations may not be related to the inhibition of ^{3}H-epibatidine binding observed by (Peters et al. 2002) but may occur via other mechanisms. Additional studies will be necessary to fully explain inhibition of ^{3}H-epibatidine binding at potentiating dFBr concentrations and the inhibition of response amplitudes at higher dFBr concentrations.

The potentiating component of the dose response curve obtained by co-application of dFBr and 100 μM ACh produced a half-maximal potentiation at a concentration of 120 ± 0.60 nM dFBr although the true potency is likely obscured by the inhibitory component of the dose-response curve (Figure 2.3B). A maximum potentiation of 295 ± 67% (n=4) of the control trace was observed at approximately 3 μM dFBr. This is similar to the 250% potentiation observed by Sala et al (Sala et al., 2005).

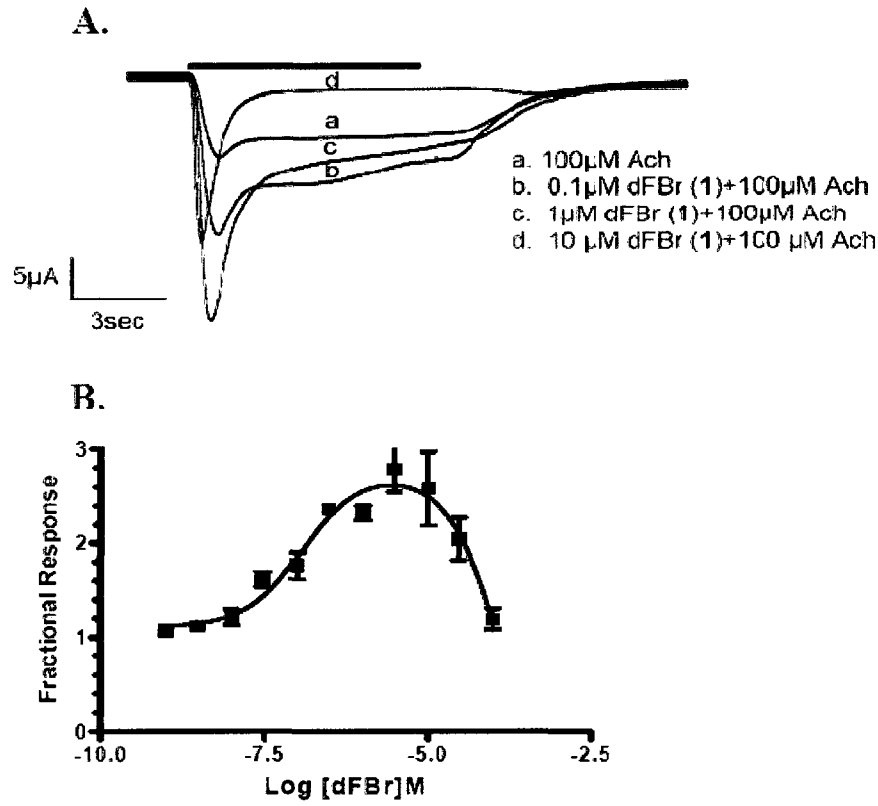

Figure 2.3: Effects of dFBr α4β2 nACh receptors A: Responses were obtained using two-electrode voltage clamp on *Xenopus* oocytes expressing human α4β2 nACh receptors. The responses elicited by each concentration of ACh/dFBr are indicated by the letters above the current trace. The time of drug exposure is indicated by the horizontal bar above the traces. Trace c (1 μM dFBr) produced a maximum potentiation of the peak response. Reduced peak amplitudes are seen at concentrations >1 μM dFBr (trace d). Peak amplitudes for each trace are: a. 14 μA, b. 24 μA, c. 37 μA, d. 25 μA) B: Dose/response curve for dFBr applied in the presence of 100 μM ACh on human α4β2 receptors. Peak amplitudes were normalized to peak currents obtained from 100 μM ACh in the absence of dFBr. Identical experimental conditions were used Figures 2A and B. The half maximal potentiation observed is 120 nM with an IC_{50} of 6.0 μM. Maximum potentiation was 295 +/- 67 % of the control trace (n=4, 13 oocytes).

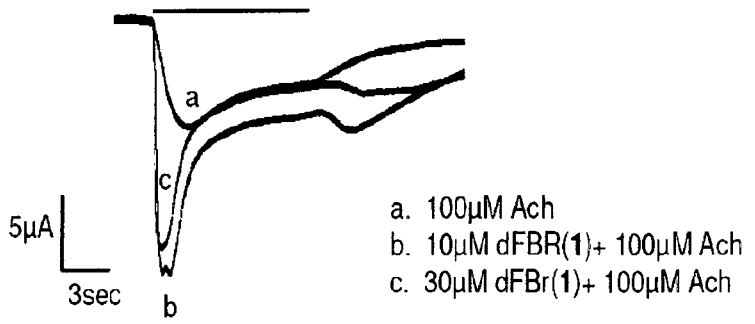

Figure 2.4: Rebound currents evident on washout of ACh and dFBr. Current traces were elicited by co-application of 100 μM ACh and dFBr (10 μM and 30 μM). Experimental conditions are identical to those shown in Figure 2.3. All three responses were obtained from a single oocyte. The horizontal black bar above the traces indicates the presence of ACh (a) or ACh + dFBr (b). Rebound currents are particularly evident in trace b.

2.4.3 Dose response curve of ACh with and without dFBr on $\alpha 4\beta 2$ receptors:

A comparison of ACh dose response curves in the presence and absence of 1 μM dFBr (Figure 2.5) showed a decrease in the apparent EC_{50} (36.8 ± 8.75 μM to 18.9 ± 3.22 μM) for ACh and a corresponding increase in the maximal response obtained, suggesting that potentiating effects may be due to binding of dFBr to a site distinct from the agonist binding site as has been proposed for other ACh potentiating ligands. The apparent efficacy of Ach increased from 1.03 +/- 0.08 without 1 μM dFBr to 3.60 +/- 0.23 with 1 μM dFBr. There was a significant increase in apparent efficacy with 1 μM dFBr (P=0.0009).

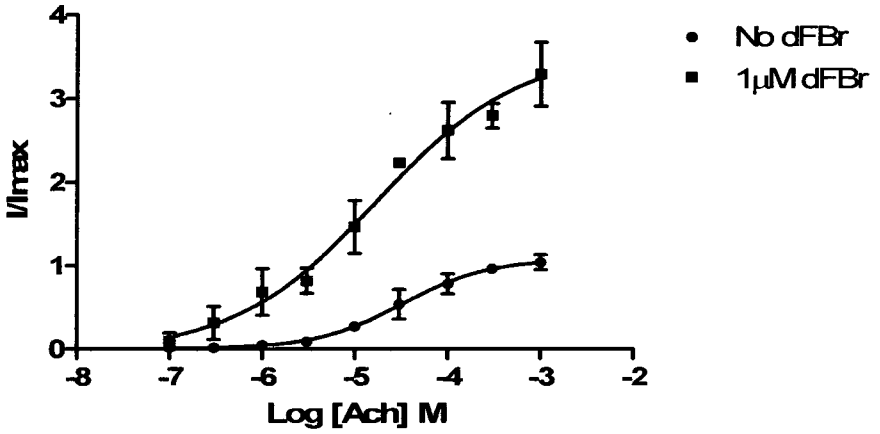

Figure 2.5: ACh dose/response curves the presence and absence of dFBr. Responses were obtained using two-electrode voltage clamp on *Xenopus* oocytes expressing human α4β2 receptors. Peak amplitudes were normalized to peak currents obtained in the presence of 100 μM ACh in the absence of dFBr. A comparison of EC_{50} values with and without dFBr shows a shift in potency of ACh for α4β2 receptors from 36.8 μM (without dFBr) to 18.9 μM (with dFBr). dFBr increased the maximal response by 360 % when compared to the maximal peak amplitudes in the absence of dFBr. The data shown reflect at least 4 repeated evaluations for each point on a minimum of 6 oocytes (n=4).

2.4.4 Action of dFBr-B on α7 and α4β2 receptors:

dFBr-B was evaluated for its effects on α7 and α4β2 nACh receptors (Figure 2.6). Co-application of dFBr-B with 100 μM acetylcholine on α7 receptors produced responses that were inhibited when compared to those obtained using 100 μM acetylcholine alone (IC_{50} = 14.2 ± 2.77 μM). Inhibited response profiles were similar to those resulting from co-application of dFBr on α7 receptors; currents were decreased in amplitude with no alteration in the rate of desensitization. Co-application of dFBr-B with 100 μM ACh s on α4β2 receptors also showed inhibition of the responses (IC_{50} = 209 ± 4.12 μM). Inhibiting concentrations of dFBr-B produced responses showing increased desensitization rates and decreased peak currents similar to that obtained for high concentrations of dFBr on α4β2 receptors (Figure 2.6B). These data suggest that dFBr and dFBr-B may inhibit α4β2 responses by a similar

mechanism. Since both dFBr and dFBr-B alter desensitization rates on α4β2 receptors but not on α7 their mechanism of action may be different on each receptor subtype.

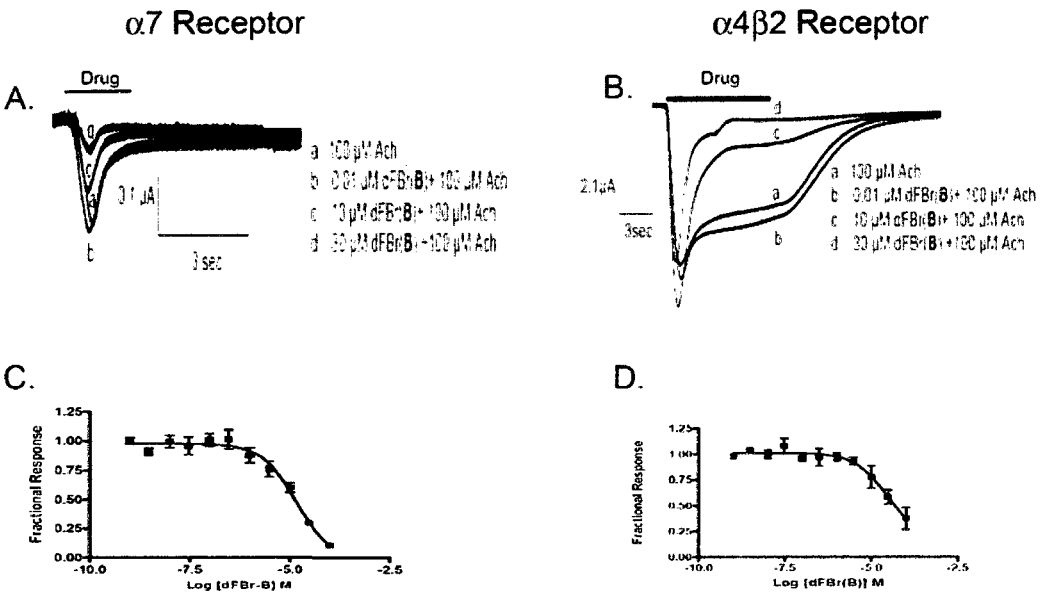

Figure 2.6: dFBr-B effects on A: α7 and B: α4β2 nACh receptors. Currents elicited by 100 μM ACh in the presence of dFBr-B using two electrode voltage clamp. dFBr-B was co-applied at varying concentrations with 100 μM ACh to *Xenopus* oocytes expressing human α4β2 or human α7 nACh receptors. C: Concentration/Response curve showing the normalized response to co-application of dFBr-B (2) and 100 μM ACh on human α7 nACh receptors. D: Concentration/Response curve of the normalized response to co-application of dFBr-B and 100 μM ACh on human α4β2 nACh receptors. Peak amplitudes were normalized to peak currents elicited by 100 μM ACh in the absence of dFBr-B (2). Data reflect at least 4 repeated evaluations for each point on a minimum of 5 oocytes (n=4).

2.5 Discussion and conclusion:

The synthetic HCl salts of dFBr and dFBr-B evaluated in this study behaved similar to the compounds obtained from *Flustra foliacea*. Two primary differences were observed. First, while Sala et al. (Sala et al., 2005) observed only potentiation, we observed both

potentiation and inhibition of peak amplitudes by dFBr on $\alpha4\beta2$ receptors and inhibition only on $\alpha7$ receptors. IC_{50} values for inhibition of acetylcholine responses on $\alpha7$ receptors agree with the K_i values determined by Peters et al. (Peters et al., 2002) in competition binding experiments. IC_{50} values obtained on $\alpha4\beta2$ receptors were significantly higher than those obtained by Peters for inhibition of ^{3}H-epibatidine binding and did not produce the decrease in overall responses typical of competitive antagonists. The change in response profiles, particularly the apparent loss of the slow desensitizing phase and the presence of rebound currents on washout of Ach-dFBr support the hypothesis that the inhibition we observed at high dFBr concentrations is due, at least in part, to open-channel block. In addition to the inhibitory effects of dFBr, we also observed an increased strength for potentiation and a slight increase in the maximal amount of potentiation obtained compared to data obtained by Sala et al (Sala et al., 2005). While these differences are not easily explained they could be due to a difference in solubility between the synthetic HCl salt used in our study and the compound purified from *Flustra Foliacea* used by Sala et al. (Sala et al., 2005) utilized DMSO to solubilize dFBr, due to its low solubility in buffer, while we used a more soluble HCl salt that may have permitted better dissolution of the compound. Decreased availability of dFBr due to DMSO solubilization or decreased concentrations of dFBr due to solubility problems would be reflected in an apparent decrease in strength for potentiation and may have prevented observation of inhibition. This conclusion is consistent with the higher potency and maximal potentiation we observed. In our preparation, dFBr showed potentiating effects at concentrations as low as 30 nM, while Sala et al (Sala et al., 2005) reported no potentiation below 3 μM. The rapid perfusion system used in our study may also have contributed to these differences. We used a modified *Xenopus* oocytes recording chamber that permits solution changes that are significantly faster than those used by Sala et al. (Sala et al., 2005) This system more accurately detects peak amplitudes prior to desensitization

since the solution exchange takes place within 200 milliseconds. Slower perfusion and exchange rates would result in smaller response amplitudes due to receptor desensitization and less potentiation would be observed at lower dFBr concentrations.

The water-soluble HCl salt of dFBr examined in this study provides a novel, $\alpha4\beta2$ receptors verses $\alpha7$ receptors, selective modulator of nACh receptors. We have confirmed that dFBr, but not dFBr-B, is capable of significantly increasing responses on $\alpha4\beta2$ receptors when co-applied with ACh. This modulation is due to an increase in apparent efficacy of ACh and not simply a shift in EC_{50} value. The increase in apparent efficacy in presence of dFBr is significant compared to affinity (P=0.0009). dFBr effects are observed at concentrations ranging from 30 nM - 100 μM although inhibition of peak amplitudes and alterations in desensitization rates are observed at concentrations greater than 3 μM. dFBr-B inhibited ACh-induced responses both on $\alpha7$ and $\alpha4\beta2$ nACh receptors.

While significant structural differences are apparent between dFBr and dFBr-B the similarity of these compounds, possibly due to the common *N*-methyl-6-bromotryptamine moiety, may enable them to mediate similar inhibitory effects on $\alpha4\beta2$ receptors. The potentiating effects of dFBr appear enhanced by structural features not common to both compounds. The selectivity of dFBr for $\alpha4\beta2$ receptors as against $\alpha7$ receptors makes it an ideal lead molecule for future studies involving modulation of $\alpha4\beta2$ nACh receptors and a possible candidate for the treatment of diseases such as Autism or Alzheimer's disease in which $\alpha4\beta2$ nACh receptors are decreased in some brain regions. Selective, potentiating ligands for a specific subtype of nAChRs would produce increased response amplitudes at nicotinic synapses without the loss of synaptic control or desensitization that results from the use of nicotinic agonists.

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CHAPTER 3: STRUCTURE ACTIVITY RELATIONSHIP STUDY OF DESFORMYLFLUSTRABROMINE[1]

3.1 Abstract:

Desformylflustrabromine (dFBr) is a positive allosteric modulator of the α4β2 subtype of neuronal nicotinic acetylcholine receptors. Here we report the results of a structure-activity relationship study for a series of dFBr analogues. Test compounds were evaluated using a two electrode voltage clamp technique and Xenopus oocytes expressing human α4β2 or α7 receptors. Out of 15 analogues tested, three potentiated responses elicited by acetylcholine on α4β2 receptors. No potentiation of acetylcholine responses was observed for any of the compounds on α7 receptors. α4β2 and α7 mediated acetylcholine responses were inhibited by the 12 analogs that did not show potentiation on either receptor. These data support our hypothesis that potentiation and inhibition are mediated through different binding sites with different structural requirements.

3.2 Introduction:

The expression of nicotinic acetylcholine receptors (nAChRs) is widespread, particularly in the central nervous system. In the brain, nAChRs are involved in fast synaptic transmission, modulation of other neurotransmitter systems and are important contributors to neuronal plasticity (Gotti et al., 2007). nAChRs are implicated in a number of neurological conditions including nicotine addiction (Mineur and Picciotto, 2008), Alzheimer's disease (Buckingham et al., 2009), Schizophrenia (Woodruff-Pak and Gould, 2002), Parkinson's disease and Autism (Arias, 2006). The most widely expressed nAChRs in the CNS are the

[1] The dFBr analogues testing reported in this chapter were designed and synthesized by Dr. Richard Glennon and his group at Virginia Commonwealth University. The data present here is entire my work except for compound JSK-062, where Maegan Weltzin performed one of the four repeats of the experiment testing this compound.

heteromeric α4β2 and the homomeric α7 receptors (Gotti et al., 2006). Due to their importance in regulation of neuronal signalling and their apparent role in neurological disorders, a significant amount of effort has been made to develop nAChR ligands, particularly those that can act selectively on individual nAChR subtypes (Astles et al., 2002). Two broad types of nAChR ligands are being actively pursued. These can be classified as orthosteric ligands (ligands that bind at the ACh binding site) and allosteric ligands (ligands binding to non-ACh binding sites). Orthosteric ligands have received much attention, particularly partial agonists like Varenicline which targets α4β2 subtype of nAChRs and provides a treatment option for nicotine addiction (Gonzales et al., 2006). However, most partial agonists or agonists cause desensitization of nAChR responses (Buccafusco et al., 2009). Hence, the use of partial agonists is limited. Subtype selective partial agonists have also proved difficult to develop, probably due to the conserved nature of the orthosteric binding sites (Springer et al., 2008). Allosteric ligands that enhance the currents elicited by ACh provide an alternate approach to treating disorders of decreased nicotinic activity (decreased nicotinic tone) (Faghih et al., 2007). Given the variety of allosteric binding sites that apparently exist on nAChRs, it may also be easier to develop subtype selective allosteric rather than orthosteric ligands (Bertrand and Gopalakrishnan, 2007).

One group of allosteric ligands commonly referred to as positive allosteric modulators or "PAMs," bind to allosteric binding sites and produce an increase in the amplitude of responses to agonists (Bertrand and Gopalakrishnan, 2007). Ideally, PAMs potentiate responses in the presence of endogenous agonists and do not, by themselves, activate the receptor. This can prevent receptor desensitization or inactivation in the continued presence of the drug and preserve ACh mediation of synaptic transmission. This is in contrast to orthosteric ligands like nicotine which produce receptor up-regulation and/or desensitization (Gentry and Lukas, 2002). In pathological conditions where nicotinic signalling in the CNS is decreased, positive allosteric modulators could be thus be used to

maintain or restore normal levels of nAChR mediated synaptic transmission while maintaining ACh mediated signalling.

dFBr is a positive allosteric modulator for α4β2 receptors that exhibits only inhibitory effects on homomeric α7 nAChRs (Kim et al., 2007). Co-application of dFBr and ACh on Xenopus oocytes expressing α4β2 receptors produces a bell shaped dose response curve characteristic of a hormetic process, with potentiation observed at lower dFBr concentrations and inhibition observed at higher concentrations (Mattson, 2008). Our collaborators (Dr. Richard Glennon and Colleagues at Virginia Commonwealth University) have synthesized a synthetic version of the natural product dFBr and 15 dFBr analogues designed to identify key pharmacophoric features of this potentially novel class of α4β2 PAMs (Kim et al., 2007). The synthesis and evaluation of synthetic dFBr was discussed in Chapter 2. Of the 15 synthetic dFBr analogues synthesized, two analogues (dFBr-B & dFBr-C) are also found in *Flustra foliacea*, the source of naturally occurring dFBr.

We hypothesized that different functional groups in the chemical structure of dFBr are responsible for the potentiation versus inhibition of ACh induced responses on α4β2 receptors and the inhibition of Ach responses on α7 receptors. If this hypothesis is valid, then substitutions of different functional groups for those on the dFBr molecule will produce analogues displaying different activities for potentiation and inhibition and may produce compounds which potentiate but do not inhibit ACh responses. Identification of functional groups important for each of the two effects of dFBr begins with the "deconstruction" of the dFBr pharmacophore described here. Future studies will utilize this information to "reconstruct" the dFBr pharmacophore. Synthetic analogues of dFBr were tested by co-application with ACh on Xenopus oocytes expressing either human α4β2 or α7 nAChRs. Compounds were evaluated for their ability to potentiate and/or inhibit responses to Ach. Three analogues displayed potentiating effects similar to dFBr on α4β2 receptors and 12

produced only inhibition. Using this data we have developed a preliminary structure activity relation for the action of the dFBr class of PAMs on α4β2 receptors.

3.3 Material and Methods:

3.3.1 Materials:

Cloned human DNA (cDNA) for human α7 and α4β2 receptors were obtained from Dr. Jon Lindstrom's laboratory (Department of Neuroscience, School of Medicine, University of Pennsylvania). Individual cDNA coding for nAChR subunits were cloned into the pBud-CE4.1 vector (Invitrogen, CA) prior to mRNA synthesis. *Xenopus laevis* frogs and frog food were purchased from Xenopus Express (Homosassa, FL). Test compounds (dFBr and dFBr analogues) were synthesized by Dr. Richard Glennon's research group at the Virginia Commonwealth University School of Pharmacy, Richmond VA. Compounds were provided as dry HCl salts.

3.3.2 *Xenopus laevis* oocytes:

Xenopus laevis frogs were maintained according to approved IACUC protocols established by the University of Alaska Fairbanks. Frog surgeries were conducted using the standard operating procedure on file at the University of Alaska Fairbanks. Ovarian lobes were surgically removed from *Xenopus laevis* frogs and washed twice in Ca^{+2}-free Barth's buffer (82.5 mM NaCl / 2.5 mM KCl / 1mM $MgCl_2$ / 5 mM HEPES, pH 7.4) then gently shaken with 1.5 mg/ml collagenase (Sigma type II, Sigma-Aldrich) for 1 h at 20-25 °C. Stage IV oocytes were selected for microinjection. Synthetic cRNA transcripts for human α7 and α4β2 were prepared using the mMESSAGE mMACHINE™ High Yield Capped RNA Transcription Kit (Ambion, TX). Oocytes were injected with a total of 50 nL cRNA at a concentration of 0.2 ng/nL in appropriate subunit ratio and then incubated at 19 °C for 24 to 72 h prior to their use in voltage clamp experiments.

3.3.3 Electrophysiology:

Electrophyiological recordings were made using an automated two-electrode voltage-clamp system incorporating an OC-725C oocyte clamp amplifier (Warner Instruments, CT) coupled to a computerized data acquisition (Datapac 2000, RUN technologies) and auto-injection system (Gilson). Recording and current electrodes with resistance of 1-4 MΩ were filled with 3 M KCl. Details of the chambers and methodology employed for electrophysiological recordings have been described previously (Joshi et al., 2004). Oocytes were held in a vertical flow chamber of 200-μL volume and perfused with ND-96 recording buffer (96 mM NaCl / 2 mM KCl /1.8 mM $CaCl_2$ / 1 mM $MgCl_2$ / 5 mM HEPES; pH 7.4) at a rate of 20 ml/min. Test compounds were dissolved in ND-96 buffer and injected into the chamber at a rate of 20 ml/min using the Gilson auto-sampler injection system. Compounds were co-applied with 100 μM ACh on α4β2 receptors and 1000 μM ACh on α7 receptors. All test compounds easily dissolved in ND-96 buffer with the exception of JSK-068(**12**) which required ND-96 buffer containing 5% DMSO.

3.3.4 Receptor Expression:

α4β2 receptors can express in two stoichiometries: the high ACh affinity $(\alpha4)_2(\beta2)_3$ containing 2 α4 and 3 β2 subunits and the low ACh affinity $(\alpha4)_3(\beta2)_2$ containing 3 α4 and 2 β2 subunits. The ratio of α4 to β2 mRNA can be varied to obtain different stoichiometry receptors α4:β2 ratio of 9:1 produced low Ach affinity receptors with an EC_{50} for Ach of 58.8 μM. α4:β2 ratio of 1:9 produced low Ach affinity receptors with an EC_{50} for Ach of 1.58 μM. A 1:1 ratio of subunits produced a mixture of low and high affinity receptors with an intermediate apparent EC_{50} of 65.6 μM. We inject Xenopus oocytes with mRNA synthesized from cDNA of the pBud-CE4.1 vector containing both α4 and β2 subunits. The α4 and β2 mRNA is injected in a 1:1 proportion thus giving a mixture of the two stoichiometries of α4β2

receptors. For both α4β2 and α7 receptors, test compounds were co-applied with 100 μM ACh in concentrations ranging from 1 nM to 100 μM.

3.3.5 Data Analysis:

Curve fitting of Concentration/response data:

Concentration/response curves were fit by non-linear curve fitting and GraphPad Prism Software (San Diego, CA) using standard built-in algorithms. For IC_{50} determinations the data were fit to a single site competition model using the equation:

$$y = 1/[1 + (IC_{50}/[antagonist])^{nH}] \quad \textbf{3.1}$$

Where, nH is the Hill coefficient.

For potentiation/inhibition data obtained for α4β2 modulation by different compounds, the data was fit to a hormetic model. The hormetic model uses a bell shaped dose-response equation and simultaneously fits both the potentiation and inhibitory effects to give an EC_{50} for the potentiation component and an IC_{50} for the inhibitory component. The accuracy by which this algorithm separates these two constants is dependant on the amount of difference between them. Mathematically, this is equivalent to fitting the stimulatory and inhibitory phases simultaneously. Data are fit to the following equation:

$$I = (1 + ((Im - 1)/(1 + 10^{((logEC50-X)*nHp)}))) / (1 + 10^{((logIC50)) * nHi)}) \quad \textbf{3.2}$$

Where, I = fractional current obtained at a given ligand concentration, Im = I_{max} (maximum theoretical current obtainable by the potentiator), EC_{50} = concentration of potentiators producing I = ½ I_{max}, IC_{50} = concentration of potentiators inhibiting to ½ I_{max}, nHp = Hill slope for potentiation, nHi = Hill slope for inhibition.

3.3.6 Statistical Analysis:

Quantitative data are expressed as mean ± S.E.M Associations between the variables such as EC_{50} and IC_{50} values for the different compounds were compared. These variables were tested by using paired student's *t*-test. P values are reported thoughout the text and represent stastical differences. All statistical differences were deemed significant at the level of $P < 0.05$. N represents the number of repeat for each data point.

3.4 Results:

3.4.1 Overview:

Fifteen synthetic analogues of dFBr were evaluated for there ability to potentiate and inhibit Ach induced responses on human α4β2 and α7 receptors. Three of the 15 synthetic analogues were able to potentiate Ach induced responses on α4β2 receptors with EC_{50}s ranging from 0.120 μM to 6.7 μM. Compounds which potentiated also inhibited at concentrations greater than the peak potentiating concentration although rank potencies for potentiation and inhibition were different. Compounds with potentiating and inhibiting activities gave a hormetic response profile typified by a bell shaped dose response curve similar to that observed for dFBr (Kim et al., 2007). All compounds were capable of inhibiting ACh induced responses on α4β2 receptors with IC_{50} values ranging from 4 μM to 209 μM (Table 3.2). All compounds also inhibited Ach responses on α7 receptors although rank potencies for inhibition were substantially different for α7 versus α4β2 receptors (Tables 3.2 and 3.3). Synthetic dFBr-C and dFBr-B (naturally occurring analogues of dFBr) inhibited but did not potentiate ACh responses.

3.4.2 Potentiating Analogues of dFBr:

Sixteen compounds were synthesized by Dr. Glennon's research group including dFBr; 13 novel analogues of dFBr and two analogues that occur naturally with dFBr in

Flustra *folicea*. Of these sixteen compounds, dFBr and three of the dFBr analogues potentiated ACh responses on α4β2 receptors when co-applied with ACh (Table 3.1 and Figures 3.1 and 3.2). Test compounds were co-applied with 100 μM ACh in concentrations from 1 nM to 100 μM. The responses obtained from individual oocytes expressing α4β2 receptors were normalized to the current obtained by applying 100 μM ACh alone. All three analogues produced hormetic responses typified by a bell shaped dose response curve similar to dFBr itself (Figure 3.2). The remaining 12 analogues and the 2 naturally occurring analogue of dFBr showed inhibition but did not potentiate ACh induced responses on α4β2 receptors. Figure 3.1 shows responses obtained for all three potentiating analogues. These compounds differ at two positions; the structure of the gem di-methyl group (R_2 in Table 3.1) and bromination of the aromatic group (R_4). Overall response profiles, levels of potentiation, rise times and effects on desensitization were similar to those observed for dFBr. All three compounds showed "hump currents" on washout of dFBr although this was somewhat more pronounced for NG-088 (4) and JSK-062 (2). Hump currents are typically observed on washout of agonists if the agonist is involved in open channel block (Liu et al., 2008). This effect is due to the dissociation of an agonist from the channel block binding site prior to dissociation from the orthosteric binding site.

Figure 3.2 shows the effect of the structural changes for potentiating analogues on both the EC_{50} and IC_{50} values. Data from these curves is shown in Table 3.1. The EC_{50} values for compound **2** (JSK-062) and dFBr were not significantly different (P=0.3094). Neither was the EC_{50} values significantly different for compound **3** (NG-089) compared to dFBr (P=0.3503) or compound **4** (NG-088) compared to dFBr (P=0.4511). However, the EC_{50} values for compound **2** (JSK-062) and compound **4** (NG-088) are significantly different (P=0.0016). As The IC_{50} values of dFBr and its three potentiating analogues do not vary significantly (all values of P>0.05). The maximal potentiation observed with each compound including dFBr was in to range of 250 to 300 %.

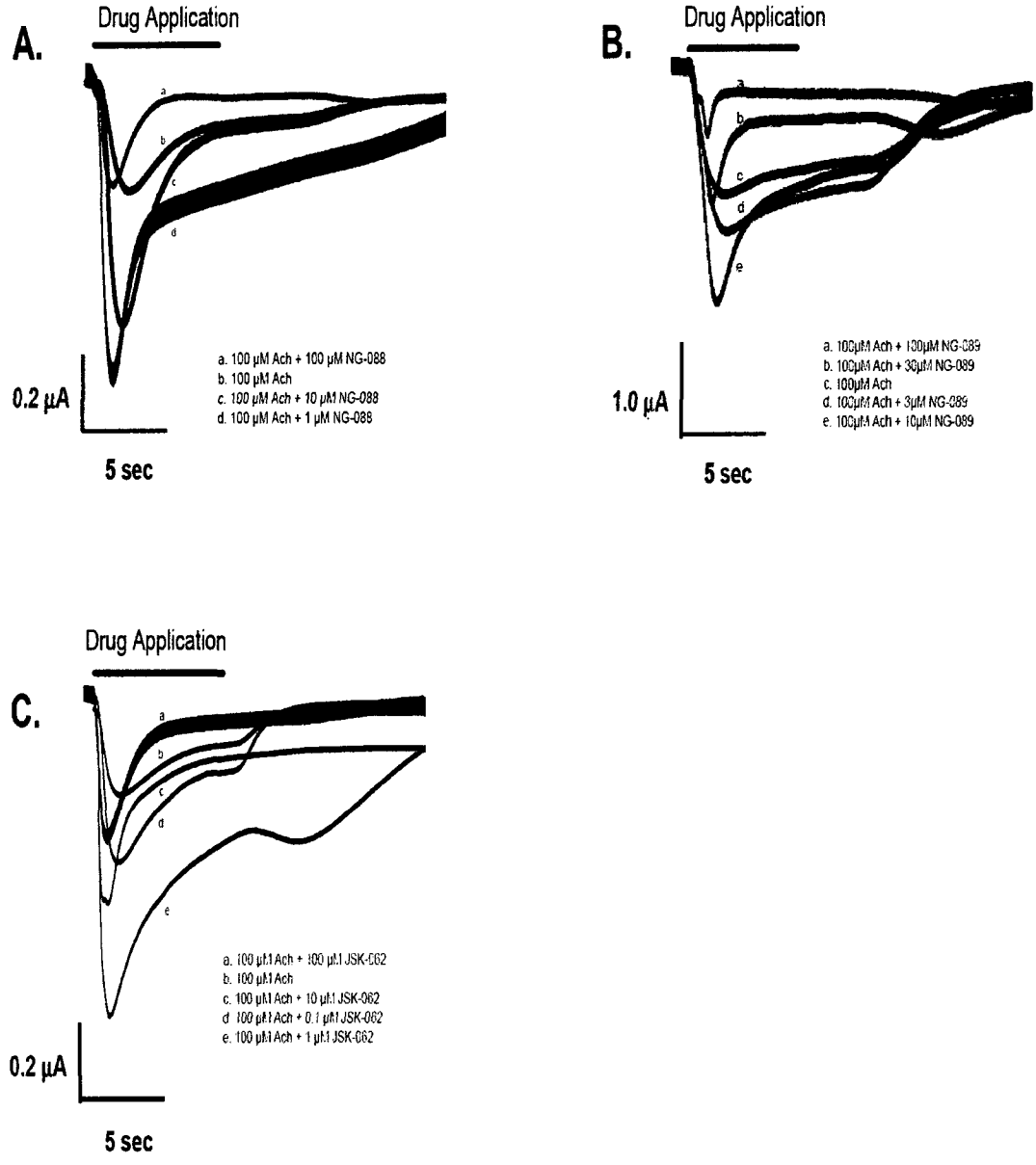


Figure 3.1: Potentiation of ACh evoked responses by dFBr analogues. Responses were elicited by application of 100 µM ACh on Xenopus oocytes expressing α4β2 nicotinic receptors. Test compounds **2, 3** and **4** were co-applied with ACh at the concentrations indicated in the figure legends. Each series of traces (A-C) were elicited from a single oocyte. Concentration/response curves for these compounds are shown in Figure 3.2 A. NG-088 (**4**). B. NG-089 (**3**). C. JSK-062 (**2**).

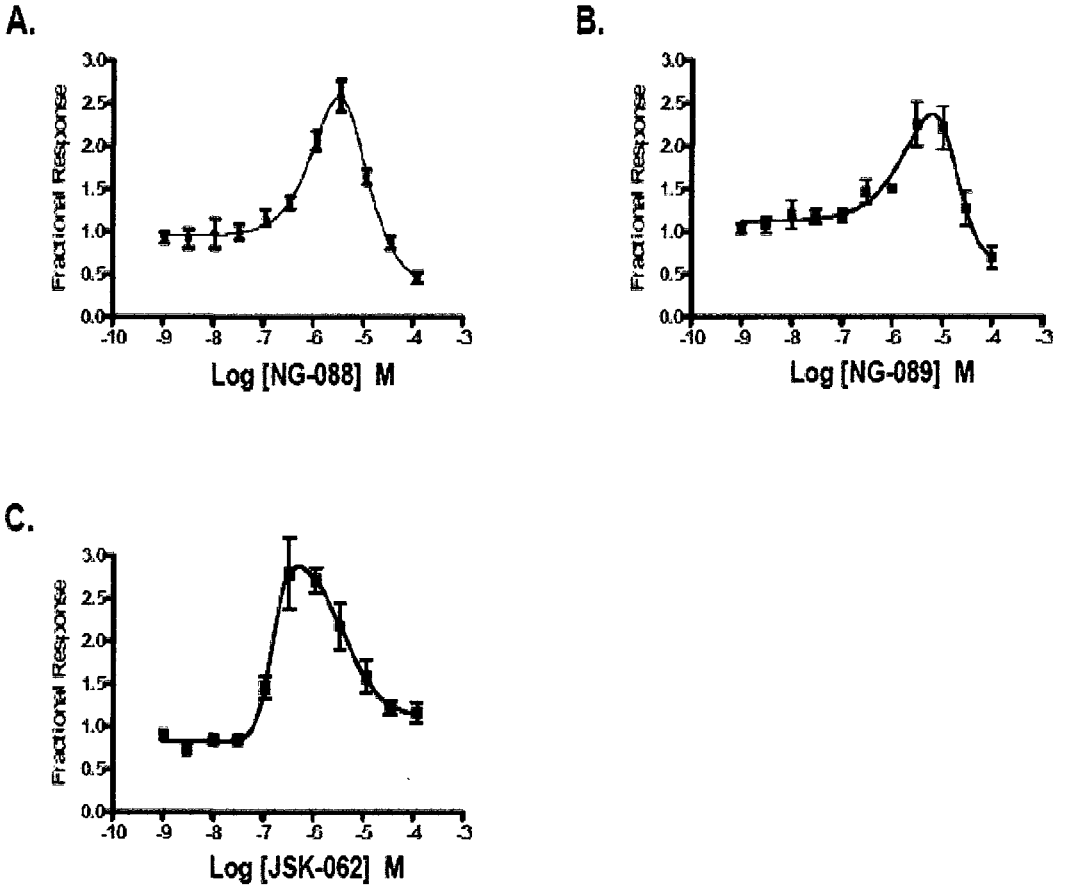
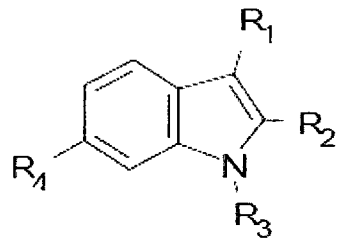


Figure 3.2: Dose/response curves for potentiating dFBr analogues. Test compounds (NG-088, NG-089 and JSK-062) were co-applied with 100 μM ACh on human α4β2 receptors. Peak amplitudes were normalized to peak currents obtained from 100 μM ACh in the absence of NG-088. Curves were fit to a hormetic model as described in the data analysis section. Data are presented in Table 3.1 **A:** Compound **4,** NG-088: The half maximal potentiation (EC_{50}) is 1.22 +/- 0.66 μM (n = 4) with an IC_{50} of 4.02 +/- 0.23 μM (n = 4). Maximum potentiation was 260% of the control trace. **B:** Compound **3,** NG-089: The half maximal potentiation (EC_{50}) is 6.77 +/- 0.35 μM (n = 4) with an IC_{50} of 17.5 +/- 1.75 μM (n = 4). Maximum potentiation is 250% of the controlled trace (100μM ACh). **C:** Compound **2**, JSK-062: The half maximal potentiation (EC_{50}) is 97.2 +/- 40.0 nM (n = 4) with an IC_{50} of 3.93 +/- 1.15 μM (n = 4). Maximum potentiation was 290% of the controlled trace (100 μM ACh). All dose-response curves, data points are mean +/- S.E.M. Lines correspond to the best fit obtained with the hormetic model bell shaped dose-response equation.

Table 3.1: Structures and activity of potentiating dFBr analogues on α4β2 and α7 receptors. Data are shown for compounds which show potentiating activity on α4β2 receptors. EC_{50} values for α4β2 receptors reflect the concentrations that give half maximal potentiation. No EC_{50} values are shown for α7 receptors since no potentiation was observed on α7 receptors. IC_{50} values for α4β2 and α7 receptors reflect the concentration required to inhibit ½ of the response to 100 μM ACh. dFBr data (**1**) is reproduced from chapter 2 for comparison. All values represents mean +/- S. E. M.



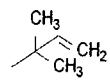
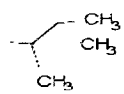
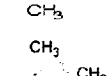
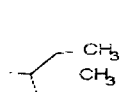
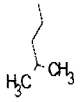
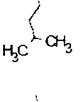
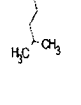
	Compound name	EC_{50} α4β2	IC_{50} α4β2	IC_{50} α7	R_1	R_2	R_3	R_4
1	dFBr	0.120 +/- 0.006 pM	6.00 +/- 0.20 μM	44.1 ± 1.25 μM*			H	Br
2	JSK-062	.0972 +/- 0.040 μM	3.93 +/- 1.15 μM	25.7 +/- 18.2 μM			H	Br
3	NG-089	6.77 +/- 0.35 μM	17.5 +/- 1.75 μM	7.74 +/- 3.99 μM			H	H
4	NG-088	1.22 +/- 0.66 μM	4.02 +/- 0.23 μM	20.6 +/- 8.09 μM			H	H

Table 3.2: Structure and Activity of non-potentiating dFBr analogues on α4β2 and α7 receptors. R_1, R_2, R_3 and R_4 refer to functional groups referenced in the structure shown in Table 3.1. dFBr-B data **(5)** is reproduced from chapter 2 for comparison. All values represents mean +/- S. E. M.

	Compound name	IC_{50} α4β2	IC_{50} α7	R_1	R_2	R_3	R_4
5	dFBr-B	209 +/- 4.00 μM	14.2 ± 2.77 μM*		H		Br
6	dFBr-C	6.68 +/- 1.10 μM	6.92 +/- 2.54 μM		H		Br
7	JSK-039	40.1 +/- 4.72 μM	30.1 +/- 2.41 μM		H	H	Br
8	JSK-067	71.1 +/- 13.9 μM	21.6 +/- 2.57 μM			H	Br
9	JSK-073	18.5 +/- 4.75 μM	44.2 +/- 19.2 μM			H	H
10	JSK-095	3.53 +/- 1.35 μM	29.5 +/- 15.0 μM			H	Br
11	JSK-065	45.1 +/- 19.5 μM	131 +/- 20.2 μM		H	H	Br
12	JSK-068	42.9 +/- 12.7 μM	20.7 +/- 1.32 μM			H	Br
13	JSK-076	46.6 +/- 16.1 μM	34.5 +/- 21.5 μM		H	H	H
14	JSK-096	15.7 +/- 4.10 μM	13.2 +/- 7.23 μM			H	Br
15	JSK-090	91.1 +/- 33.7 μM	43.7 +/- 13.6 μM		H		Br
16*	JSK-092	57.9 +/- 15.9 μM	155 +/- 40.4 μM		H		H

3.4.3 Inhibition of ACh induced responses on α4β2 by dFBr analogues

All 15 analogues, including the two naturally occurring analogues 5 (dFBr-B) and 6 (dFBr-C) act as inhibitors of α4β2 receptors. Compounds that potentiated (previous section) also inhibited Ach induced responses at higher concentrations. IC_{50} values for individual analogues varied over a range from 3.53 μM to 209 μM (Tables 3.1 and 3.2). Dose response curves for compounds showing only inhibition of ACh responses on α4β2 receptors are shown in Figure 3.3 and data obtained from these curves is presented in Table 3.2.

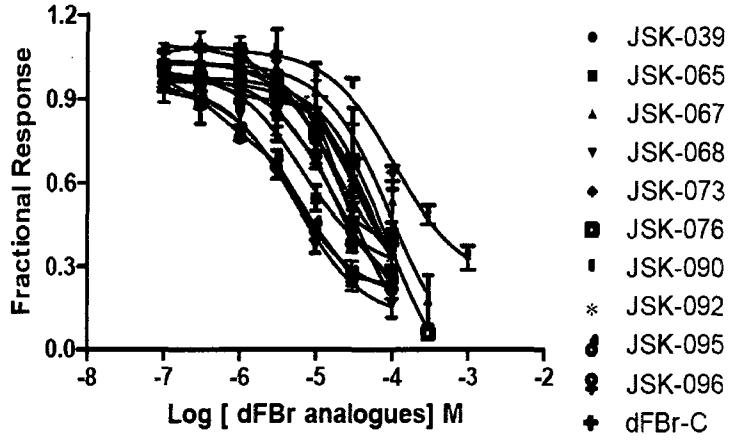


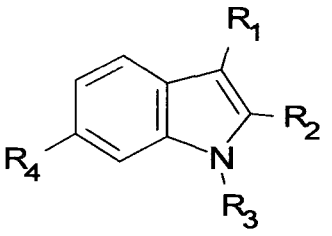
Figure 3.3: Dose/response curve for all the dFBr analogues (except dFBr-B) that inhibit α4β2 receptors without potentiation. All dose-response curves, data points are mean +/- S.E.M of at least 4 repeated evaluations (n = 4). Lines correspond to the best fit obtained with the single site competition model equation.

3.4.4 Effect dFBr analogues on α7 receptors:

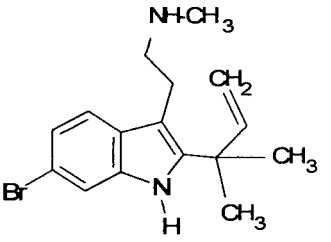
All 16 compounds inhibit responses of α7 receptors to 100 μM ACh over a potency range of 6.92 to 131 μM. IC_{50} values for inhibition of responses to 100 mM ACh on α7 receptors are shown in column 5 of Table 3.1 and column 4 in Table 3.2, column potencies of most compounds were similar on both α7 and α4β2 receptors and changes in IC_{50}

resulting from structural changes on the ligand were equivalent in most cases. No potentiation of α7 responses was observed for any of the 16 compounds tested.

3.5 Discussion:



Core Structure



dFBr

R_1 = terminal amine group;

R_2 = geminal dimethyl group;

R_3 = ring nitrogen group;

R_4 = 6-Bromo group;

Figure 3.4: Core Structure of desformylflustrabromine

3.5.1 Important functional groups of dFBr responsible for potentiation:

Two functional groups stand out as being important in the potentiation of α4β2 receptors by dFBr analogues. These are:

1) Geminal-dimethyl group at R_2 position:

All the compounds that are potentiators of α4β2 receptors have a geminal (gem)-dimethyl group at position the R_2 position (Table 3.1). Any drastic changes in the structure of this side group compound **8** (JSK-067) or the loss of it, compound **7** (JSK-039) leads to loss

of potentiation (Table 3.2). The EC_{50} values for dFBr and compound **2** (JSK-062) are not significantly different (P=0.3094). dFBr and compound **2** (JSK-062) differ only in the structure of the gem-dimethyl group in which the double bond is removed in compound **2** (JSK-062) relative to dFBr. Removal of the double bond would increase rotational freedom of this group. Similarly, the IC_{50} values for compound **2** (JSK-062) was increased slightly but not significantly compared to dFBr (P=0.773). Our data from analogue testing generally suggest that the gem-dimethyl is essential for the potentiating action of dFBr class of compounds. As long as the integrity of the structure of gem-dimethyl group is retained there is no significant change in either EC_{50} or IC_{50} values of the potentiating analogues.

2) Bromine at R_4 position:

Removal of the bromine at the R4 position produced a significant change in EC_{50} when compared to compounds identical at all other positions (dFBr compared to compound **3** (NG-089) and compound **2** (JSK-062) compared to compound **4** (NG-088)). The EC_{50} value for Compound **3** (JSK-089) was increased slightly but significantly compared to dFBr (P= 0.0002). However, the EC_{50} values for Compound **2** (JSK-062) was significantly lower compared to Compound **4** (NG-088) (P=0.0016). Compound **2** (JSK-062) and compound **4** (NG-088) are identical except for the lack of the Br at position R_4 in compound **4** (NG-088). The significant decrease in EC_{50} resulting from removal of the bromine indicates an involvement of this group in interaction with the receptor. This is likely due to a change in the electronics of the aromatic group of dFBr. While we have not evaulated any analogues where the Br atom is replaced by other halogens it is likely that such changes at 6-position may change the potentiating activity of dFBr and its analogues. Such analogs are planned during the future "reconstruction" phase of this project.

3.5.2 Importance of functional groups of dFBr for inhibition of α4β2 and α7 receptors:

The testing of dFBr analogues also aided in defining the structure activity relationship for the inhibitory action of dFBr analogues on α7 and α4β2 receptors. For this purpose all three side groups, namely the R_1 - terminal amine group, R_2 - geminal-dimethyl group and bromine at R_4 position, in the structure of dFBr were found to be important. Each of these side groups are discussed below:

1) R_1 - terminal amine group:

a) α4β2 receptors: Conversion of the primary amine at R_1 of compound **7** (JSK-039) to a secondary amine to make compound **11** (JSK-065) produced no significant change in IC_{50} value (7: 40.1 +/- 4.72 μM, 11: 45.1 +/- 19.5 μM, P=0.0726) and a slight, but significant decrease in IC_{50} was observed for conversion of the primary amine in compound **8** (JSK-067) to a tertiary amine to make compound **12** (JSK-068) (**8: 71.1** +/- 13.9 μM, **12**: 42.9 +/- 12.7 μM, (P=0.0308). Conversion of the primary amine to an amino group in compound 8 (JSK-067) to an amino group to make compound **14** (JSK-096) decreased the IC_{50} to 15.7 +/- 4.10 μM making a significant decrease in IC_{50} (P=0.0224). Thus, increasing the organic substituents on the amine nitrogen produced an apparent progressive increase in IC_{50} values.

b) α7 receptors: Conversion of the primary amine at R_1 in compound **7** (JSK-039) to a secondary amine in compound **11** (JSK-065) produces a significant change in IC_{50} for inhibition of α7 receptors (**7**: 30.1 ± 2.41 μM, **11**: 131 ± 20.2 μM, P=0.0177). This change of 4 fold is in contrast to no change in IC_{50} on the α4β2 receptor. No significant change in IC_{50} (P=0.4400) was observed when the secondary amine of compound **8** (JSK-067) is converted to a quaternary amine compound **12** (JSK-068).

2) R_2 – Geminal - dimethyl group and R_3 – Ring nitrogen group:

a) α4β2 receptors: A comparison of inhibitors differing at position R_3 reveals the importance of this group for inhibition of α4β2 receptors. Compounds **5** and **6** (dFBr-B and dFBr-C) differ only in the presence of propylene rather than a propyl group at R_3 in compound **5** (dFBr-B) versus compound **6** (dFBr-C). This change produces a significant decrease in IC_{50} from 209 +/- 4.0 μM for compound **5** to 6.68 +/- 1.10 μM for compound **6** (dFBr-C) on α4β2 receptors (P=0.0060). Removal of this propyl group entirely as in compound **7** (JSK-039) results in an IC_{50} value that is intermediate between that of compound **5** (dFBr-B) and compound **6** (dFBr-C) (40.1 +/- 4.72 μM). Removal of the propyl from R_3 and addition of a propyl group at R_2 to make compound **8** (JSK-067) does not alter the IC_{50} compared to complete removal of the group from R_3 (IC_{50} = 3.53 +/- 1.35 μM).Thus, for potent inhibition of α4β2 receptors, a propyl group appears necessary for potency at R_3 but addition of a similar group to R_2 has little effect and does not restore the function lost by the removal of the group from R_3.

b) α7 receptors: A comparison of IC_{50} for dFBr and **6** (dFBr-C) shows that the addition of a propyl group at position R_3 in dFBr results in a decrease in IC_{50} (dFBr: 44.1 ± 1.25 μM, **6**: 6.92 ±. 2.54 μM, P= 0.0020). This decrease is equivalent to that observed for α4β2 receptors with similar potencies for these compounds on both receptors. Addition of the propyl group to R_2 does not alter the IC_{50} when compound **7** (JSK-039) is compared to compound **8** (JSK-067). These data are similar to that observed for the α4β2 receptor, however the α7 receptor appears more resistant to the conversion of the propyl to a propylene group at R_4 with no significant change in IC_{50} observed for compound **5** (propylene) versus compound **6** (propyl). This is compared to a 31 fold decrease in IC_{50} on inhibition of α4β2 receptors.3)

3) R_4 – 6-Bromo group:

a) $\alpha4\beta2$ receptors: Unlike potentiation, inhibition was less affected by removal of the bromine group at position R_4. Removal of the bromine from dFBr as in compound **3** (NG-089) produces only about a three fold increase IC_{50}. Although this change is significant (P= 0.0372). A comparison of Compound **2** (JSK-062) and compound **4** (JSK-088) which also contain a change at R4 shows no significant change in IC_{50} (P=0.9144). Similar effects are seen with other pairs of analogues that have similar changes in the R_4 bromine. Compound **8** (JSK-067) is structurally similar to compound **9** (JSK-073), except for the absence of bromine at R_4 in compound **9**. The presence of bromine significantly increases the IC_{50} values of compound **8** by four fold to 71.1 +/- 13.9 μM from 18.5 +/- 4.75 μM for compound **9** (P= 0.0387).

b) $\alpha7$ receptors: The presence of bromine at the R_4 position appears to have little if any effect on IC_{50} values for inhibition of $\alpha7$ receptors. Compound **3** (NG-089), which is identical in structure to dFBr but lacks the R_4 bromine is about 5.5 fold more potent than dFBr (P=0.0374). The change in IC_{50} on removal of the bromine group from dFBr is similar to that observed for $\alpha4\beta2$ receptors although in the opposite direction. Removal of the bromine from dFBr appears to enhance apparent affinity for $\alpha7$ while decreasing apparent apperant for $\alpha4\beta2$. Compounds **2** (JSK-062) and compound **4** (JSK-088) also differ with respect to the presence in compound **2** or absence in compound **4** of a bromine at R_4, but their IC_{50} values are identical. Similar effects on IC_{50} values are seen for other pairs including compound **8** (JSK-067) and compound **9** (JSK-073) as well as compound **11** (JSK-065) and compound **13** (JSK-076).

3.7 Conclusion:

Taken together, the data from functional testing of dFBr analogues has lead to the development of a general SAR for this class of positive allosteric modulator for $\alpha4\beta2$ receptors. We had originally hypothesized that different functional groups in the chemical structure of dFBr are responsible for the potentiation versus inhibition of acetylcholine induced responses on $\alpha4\beta2$ receptors. We have identified the important groups and their role in potentiation and inhibition of $\alpha4\beta2$ receptors. The gem-dimethyl group in dFBr and its analogues is essential for the potentiating effect. Other functional groups do not seem essential for potentiation and have varying effect on inhibition of $\alpha4\beta2$ receptors. It is likely that inhibition and potentiation occur due to binding of dFBr and its analogues on different binding sites present on $\alpha4\beta2$ receptors. dFBr and its potentiating analogues represent a unique class of positive allosteric modulators for heteromeric $\alpha4\beta2$ subtype of neuronal nAChRs.

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CHAPTER 4: COMPARATIVE ANALYSIS OF THE EFFECTS OF POSITIVE ALLOSTERIC MODULATORS ON HUMAN NICOTINIC ALPHA4 BETA2 RECEPTORS EXPRESSED IN XENOPUS OOCYTES.[1]

4.1 Abstract:

A number of allosteric modulators have been identified that are able to potentiate acetylcholine (ACh) responses on neuronal nicotinic acetylcholine receptors (nAChR). These compounds can be grouped into broad structural classes typified by ionic zinc (Zn^{2+}), physostigmine and 17-β-Estradiol (17-BE). Each class of modulator appears to bind to unique binding sites to produce potentiation (Hsiao et al., 2006; Paradiso et al., 2001; Schrattenholz et al., 1993). Recently, desformylflustrabromine (dFBr), a metabolite from *Flustra folicea* was found to potentiate nAChRs and may represent a new structural class of allosteric modulators for these receptors. Unlike other known potentiating agents, dFBr shows high selectivity within the nAChR family for the α4β2 subtype compared to α7 receptors (Kim et al., 2007). Our laboratory, in conjunction with Dr. Richard Glennon (Virginia Commonwealth University, College of Pharmacy) have synthesized and characterized the synthetic form of dFBr (Kim et al., 2007). In this study, we evaluate dFBr's action on α4β2 receptors along with Zn^{2+}, physostigmine and 17-BE. The goal of this study is to first compare all four allosteric modulators independently within the same system and secondly to determine the effects of co-application of each modulator with dFBr. Since some data is available regarding the site of action of other modulators, a comparison of dFBr with these modulators will serve as a guide and starting point to further exploration of dFBr's mechanism and binding site through Site directed mutagenesis.

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[1] The dFBr used for this study was synthesized Dr. Richard Glennon and his group at Virginia Commonwealth University.

Two electrode voltage clamp experiments were conducted using Xenopus oocyte expressing human α4β2 receptors. dFBr potentiated both high and low ACh affinity α4β2 receptors, similar to Zn^{2+} by increasing the current elicited by ACh even at saturating concentrations of agonist. Unlike dFBr however Zn^{2+} potentiated only low ACh affinity receptors (Moroni et al., 2008).

Physostigmine decreased the I_{max} values at saturating concentrations of agonist along with a decrease in apparent affinity. When co-applied with dFBr, physostigmine induced a proportional decrease in I_{max} value and apparent affinity, the effects of dFBr/physostigmine appeared additive. This is consistent with independent mechanisms and sites of action for these two compounds. In contrast, neither Zn^{2+}, 17-BE, nor dFBr significantly increase the apparent affinity of ACh on α4β2 receptors. When co-applied with dFBr, Zn^{2+} produced no significant additional effect on the changes in apparent efficacy but significantly altered apparent affinity compared to dFBr alone. No changes in these parameters were observed in these parameters on co-application of 17-BE and dFBr. The lack of additive effects of dFBr with 17-BE could indicate similar mechanisms of action and possibly similar binding domains for these compounds. These data represent important leads for the identification of the dFBr binding domain as they will guide future site directed mutagenesis studies.

4.2 Introduction:

Nicotinic acetylcholine receptors (nAChRs) are proteins present on the cell membrane that respond to the neurotransmitter ACh. nAChRs along with 5-HT_3R, GABA(A), GABA(C) and Glycine receptors form a super-family of Cys-loop ligand gated ion channels. In the brain, nAChRs are involved in fast synaptic transmission and neuronal plasticity. They have been implicated in a number of neurological conditions including nicotine addiction, Alzheimer's disease, Schizophrenia, Parkinson's disease and autism (Gotti et al., 2006).

Positive allosteric modulators (PAMs) are rapidly being developed for neuronal nAChRs and may represent a promising new approach for treating disorders involving these receptors (Gopalakrishnan et al., 2007).

A number of allosteric modulators have been identified that are able to potentiate ACh responses on neuronal nAChR. These compounds can be grouped into broad structural classes typified by Zn^{2+}, physostigmine (see figure 1.10(B) for structure of physostigmine) and 17-β-Estradiol (17-BE) (see figure 4.5 structure of 17-BE). Each class of modulator appears to bind to unique binding sites to produce potentiation (Hsiao et al., 2006; Paradiso et al., 2001; Schrattenholz et al., 1993).

The acetylcholinesterase inhibitors galantamine and physostigmine were among the first compounds demonstrated to have an allosteric modulatory effect on neuronal nAChRs (Maelicke et al., 2000). Physostigmine was shown to potentiate α4β2 receptors at an ACh concentration of 1 μM (Smulders et al., 2005). Photo-affinity labelling studies in torpedo nAChR determined that a region located near K125 of the α-subunit is the likely binding site for physostigmine and this binding site is distinct from the ACh orthosteric binding site (Schrattenholz et al., 1993).

The steroidal ligand 17-BE modulates α4β2 receptors by binding to the short C-terminal tail of the α4 subunit of this receptor (Paradiso et al., 2001). At the single channel level, 17-BE potentiation results from an increase in opening probability of the α4β2 receptor channel. (Curtis et al., 2002).

Zn^{2+}, another allosteric modulator of nAChRs, potentiates both β2 and β4 containing subtypes (Hsiao et al., 2001). Receptors expressed with a stoichiometry of 3 α4 and 2 β2 subunits, $(\alpha4)_3(\beta2)_2$ receptors, show low affinity for ACh (60 μM) and are potentiated by Zn^{2+} while those expressed with a stoichiometry of 2 α4 and 3 β2 subunits, $(\alpha4)_3(\beta2)_2$ receptors, show high affinity for ACh (2 μM) and are not potentiated by Zn^{2+}. The apparent selectivity of Zn^{2+} for low versus the high affinity subtypes led to the suggestion that the α4+/α4- subunit

uniquely present on the low affinity subtype is responsible for Zn^{2+} potentiation (Moroni et al., 2008). Using site directed mutagenesis and the substituted cysteine accessibility method (SCAM) key residues within the β2+/α4- interface of the α4β2 receptor have been identified as critical for Zn^{2+} potentiation (Hsiao et al., 2006). Single channel studies have determined that Zn^{2+} potentiates α4β4 receptors by increasing burst frequency of the receptor (Hsiao et al., 2008).

Desformylflustrabromine (dFBr) a metabolite isolated from the marine bryozoan *Flustra foliacea* has also been shown to potentiate the α4β2 subtype of nAChR (Sala et al., 2005). It does not potentiate ACh responses on other nAChR subtypes including α7, α3β4 and α3β2 (Sala et al., 2005). Our laboratory and our collaborators in Dr. Richard Glennon's Laboratory (Virginia Commonwealth University, School of Pharmacy) have developed a synthetic HCL salt of dFBr and have confirmed the action of this compound as an allosteric potentiator for α4β2 (Kim et al., 2007). This compound is unique in its ability to potentiate α4β2 receptors while inhibiting ACh induced responses on homomeric α7 receptors. Co-application of increasing dFBr concentrations (1 nM to 100 μM) along with a fixed concentration of ACh produced a hormetic dose response curve on α4β2 receptors with the receptor responses potentiated at low concentrations of dFBr and inhibited at higher concentrations of the drug (Kim et al., 2007). A standard sigmoidal inhibition curve is observed under these conditions for α7 receptors.

We have hypothesized that the effects of dFBr are due to its binding at a unique binding site that is different from the binding site for other α4β2 receptor allosteric modulators. In this study we evaluated the modulatory actions of the three classes of positive allosteric modulators (PAMs) on human α4β2 receptors expressed in Xenopus oocytes. We compare the characteristics of modulation by physostigmine, 17-BE and Zn^{2+} to the effects produced by dFBr on both α4β2 and α7 receptors. Although another class of recently

identified PAMs, represented by (2-amino-5-keto) thiazole, are also active on α4β2 receptors (Broad et al., 2006) these compounds are not commercially available, and thus could not be included in this study.

In our study, dFBr potentiated both high and low ACh affinity α4β2 receptors. This is in contrast to the effects of Zn^{2+} which potentiates only the low affinity subtype. When co-applied with dFBr, Zn^{2+} did not produce any increase in potentiation compared to dFBr alone. This may suggest similar mechanisms but perhaps different binding site locations for dFBr and Zn^{2+}. Similar to Zn^{2+}, 17BE did not produce and additional potentiation when applied with dFBr; again suggesting either similar mechanism and/or binding sites. In contrast to dFBr, physostigmine decreased the I_{max} values at saturating concentrations of agonist along with a decrease in apparent affinity. When co-applied with dFBr, physostigmine produced a decrease in I_{max} similar to the decrease observed in the absence of dFBr. The apparent affinity also decreased a similar amount whether or not dFBr was present. These data indicate additive actions of dFBr and physostigmine mediated by different mechanisms and sites of action. Thus, possible binding sites for dFBr are at the plus face of the β2 subunit that would overlap with the Zn^{2+} binding site or on the c-terminal region overlapping the 17-BE site.

4.3 Materials and Methods:

4.3.1 nAChR subunit cDNA:

The cDNA for human α4β2 receptors were obtained from Dr. Jon Lindstrom's laboratory. Individual subunit cDNA was cloned into the pBud-CE4.1 (Invitrogen, Carlsbad, CA) vector prior to mRNA synthesis. The cDNA for α4 and β2 subunits were cloned individually in to the pcDNA3.1/Zeocin and pcDNA3.1/Hygromicin (Invitrogen, Carlsbad, CA) vectors respectively. For obtaining 1:1 expression both α4 and β2 subunits, cDNA from

vector pBud-CE4.1 was injected. To obtain expression in different stoichiometries mRNA of α4 and β2 subunits in the pcDNA3.1/Zeocin and pcDNA3.1/Hygromicin vectors respectively was used.

4.3.2 Test Compounds:

Acetylcholine (ACh), physostigmine, 17-Beta estradiol (17-BE) and Zn^{2+} in form of $ZnCl_2$ was obtained from (Sigma Aldrich, St. Louis, MO). Desformylflustrabromine (dFBr) was synthesized by Dr. Richard Glennon's laboratory as the HCL salt according to protocols described elsewhere (Kim et al., 2007).

4.3.3 Synthesis of mRNA and Injection into Xenopus oocytes:

Expression vectors containing the appropriate cDNA were linearized using the restriction endonuclease FSP-1 for all three vectors. Synthetic cRNA transcripts for human α4β2 receptor were prepared using the mMESSAGE mMACHINE™ High Yield Capped RNA Transcription Kit (Ambion, TX). For production of high and low affinity α4β2 receptors we used the method of Zwart et. al. in which neuronal nAChR α4 and β2 receptor coding mRNAs are injected into *Xenopus laevis* oocytes at different ratios of α4 to β2 (9:1, 1:1, and 1:9) (Zwart and Vijverberg, 1998). The EC_{50} values they obtained from the α4:β2 ratios 9:1, 1:1, and 1:9 were 58.8 μM, 65.6 μM and 1.84 μM respectively (Zwart and Vijverberg, 1998).

Xenopus laevis frogs and frog food were purchased from Xenopus Express (Homosassa, FL). Ovarian lobes were surgically removed from *Xenopus laevis* frogs, washed twice in Ca^{+2}-free Barth's buffer (82.5 mM NaCl / 2.5 mM KCl / 1 mM $MgCl_2$ / 5 mM HEPES, pH 7.4) then gently shaken with 1.5 mg/ml collagenase (Sigma type II, Sigma-Aldrich) for 1 h at 20-25 °C. Stage IV oocytes were selected for microinjection. Oocytes were injected with a total of 50 nL cRNA at a concentration of 0.2 ng/nL in appropriate subunit ratios then incubated at 19 °C for 24 to 72 h prior to their use in voltage clamp experiments.

4.3.4 Electrophysiological Recording:

Recordings were made using an automated two-electrode voltage-clamp system incorporating an OC-725C oocyte clamp amplifier (Warner Instruments, CT) coupled to a computerized data acquisition (Datapac 2000, RUN technologies) and auto injection system (Gilson). Recording and current electrodes with resistance of 1-4 MΩ were filled with 3 molar KCl. Details of the chambers and methodology employed for electrophysiological recordings have been described earlier (Joshi et al., 2004). Briefly, oocytes are held in a vertical flow chamber of 200 μL volume and perfused with ND-96 recording buffer (96 mM NaCl / 2 mM KCl /1.8 mM $CaCl_2$ / 1 mM $MgCl_2$ / 5 mM HEPES; pH 7.4) at a rate of 20 ml/min. All test compounds were dissolved in ND-96 buffer. Dissolution of 17-BE required the use of 0.15% DMSO in ND-96 buffer. Test compounds were injected into the chamber at a rate of 20 ml/min using the Gilson auto-sampler injection system. For experiments involving application of both modulators ACh and the appropriate modulator were mixed prior to injection.

4.3.5 Data and statistical Analysis:

Concentration-response curves were fit by non-linear curve fitting and GraphPad Prism Software (San Diego, CA) using standard built-in algorithms. For EC_{50} determinations data obtained from electrophysiological experiments were fit to the Hill equation:

$$I = I_{max} / (1 + EC_{50} / [A]^n) \qquad \textbf{4.1}$$

Where, I is the current at a given agonist concentration, I_{max} is the maximal current obtained at saturating agonist (ACh) concentration, EC_{50} is the agonist concentration that elicits a half-maximal current, and n is the Hill coefficient.

Quantitative data are expressed as mean ± S.E.M. Paired student's-*t* test and one way Analysis of Variation (ANOVA) with post-hoc Dunnett's multiple comparison tests were used to compare data from electrophysiological experiments. Variables such as the EC_{50}, Hill

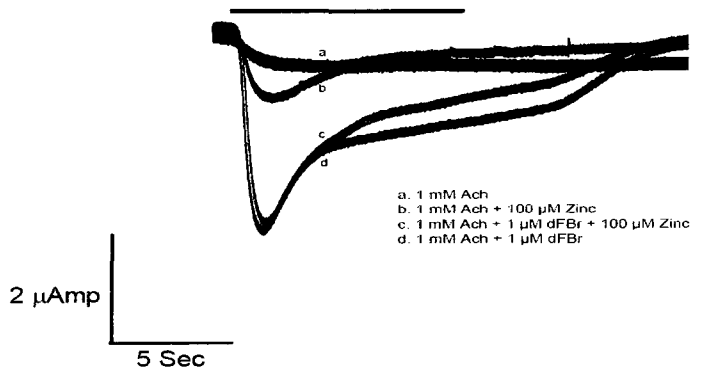
coefficient and I_{max} values were compared. P values are reported throughout the text and significance was set as $P<0.05$. N represents the number of repeats for each experimental data point and unless otherwise stated n was four or more for each data point.

4.4 Results:

4.4.1. Zinc and dFBr:

Figure 4.1A below shows responses obtained from α4β2 expressing oocytes on co-application ACh with 100 μM Zn^{2+} or 1 μM dFBr. Zn^{2+} and dFBr concentrations were chosen based on the concentration producing a peak potentiation of the hormetic response curve (Hsiao et al., 2001). Both Zn^{2+} and dFBr potentiated ACh responses on α4β2 receptors although the maximum change in I_{max} for Zn^{2+} was only 147% compared to 355% with dFBr (Table 4.1). Despite the change in response amplitudes both Zn^{2+} and dFBr produces similar effects on the overall response kinetics; increasing the peak with little apparent change in desensitization rates. Zn^{2+} did not produced a significant change in the ACh EC_{50} (P= 0.1770) and dFBr produced only a slight change in ACh EC_{50} which was not significant (P= 0.0970). Co-application of Zn^{2+} and dFBr produced no additional change in potentiation compared to the strongest potentiator dFBr alone (Table 4.1) but did produce significant changes in both EC_{50} (P=0.05) and Hill Coefficient (P=0.05). This may be a cumulative effect of slight but statistically significant changes for both dFBr and Zn^{2+} together.

A)



B)

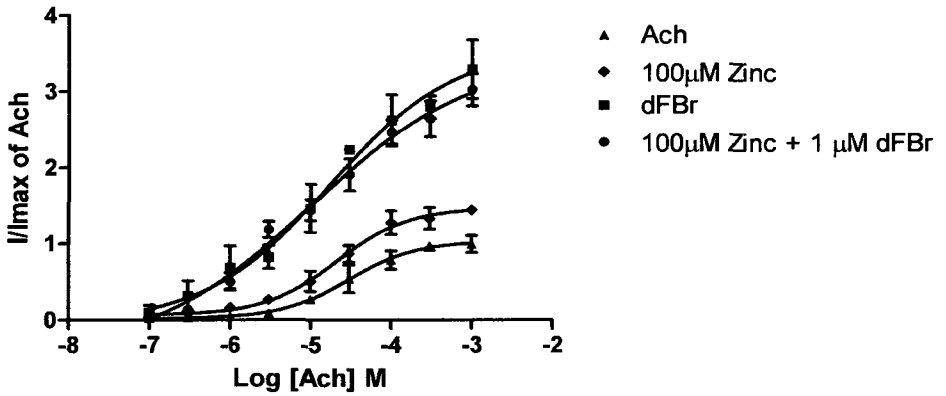


Figure 4.1: The effect of Zn^{2+} and dFBr on $\alpha4\beta2$ receptors.
Responses shown in A and B were obtained using Xenopus oocytes and two-electrode voltage clamp. Membrane potentials were clamped at -60 mV. Peak current amplitudes for each point were normalized to peak currents obtained in the presence of 1 mM ACh without dFBr or Zn^{2+} present. Data are tabulated in Table 4.2. A: Responses obtained from Xenopus oocytes expressing $\alpha4\beta2$. Membrane potential was clamped at -60 mV. Traces a, b, c and d were obtained from different oocytes and were normalized to the trace obtained from application of 1 mM Ach. B. ACh concentration/response curve with dFBr, Zinc and dFBr with Zinc on human $\alpha4\beta2$ receptors. All dose-response curves, data points are mean +/- S.E.M. Each data point on all the four curves were evaluated at least 4 times on a minimum of 2 oocytes for each curve (n=4). Lines correspond to the best fit obtained with the Hill equation.

Table 4.1: EC_{50} for ACh activation, I_{max} and Hill slopes for responses obtained by zinc, dFBr and by co-application of zinc with dFBr on mixed stoichiometry α4β2 receptors. The I_{max} was normalized to that obtained for 1 mM ACh on the same oocyte. All values are mean +/- S.E.M.

	EC_{50} (μM)	**Normalized I_{max}**	**Hill slopes**
ACh	36.8 +/- 8.75μM	1.03 +/- 0.04	1.07 +/- 0.17
1 μM dFBr	18.9 +/- 3.22 μM	3.57 +/- 0.23	0.60 +/- 0.01
100 μM Zinc	22.4 +/- 3.47 μM	1.47 +/- 0.05	0.99 +/- 0.14
1 μM dFBr + 100 μM zinc	10.1 +/- 5.2 μM	3.55 +/- 0.21	0.43 +/- 0.05

The effects of dFBr and Zn^{2+} on EC_{50} and Hill slope when co-applied may indicate an additive effect of the two compounds although this additive effect was not evident in their effects on potentiation. To explore the binding site interactions more fully, we evaluated the action of dFBr on different stoichiometries of the α4β2 receptor. Previous studies have shown that Zn^{2+} potentiates the low affinity α4β2 subtype but it is unable to potentiate the high affinity subtype (Moroni et al., 2008). Figure 4.2 shows the effects of dFBr on the two different stoichiometries of α4β2 receptors. The potentiated responses are the same for both high and low affinity receptors although the high affinity potentiated response appears sharper, possibly due to increased rate of desensitization. The increase in I_{max} produced by co-application of dFBr is significantly more for the high affinity receptor compared to the low affinity receptor (Table 4.2 - 504% vs. 274%) (P= 0.0007). It is evident from these data that dFBr is capable of potentiating both high and low affinity α4β2 receptors in contrast to Zn^{2+} which appears to potentiate only the low affinity receptor (Moroni et al., 2008).

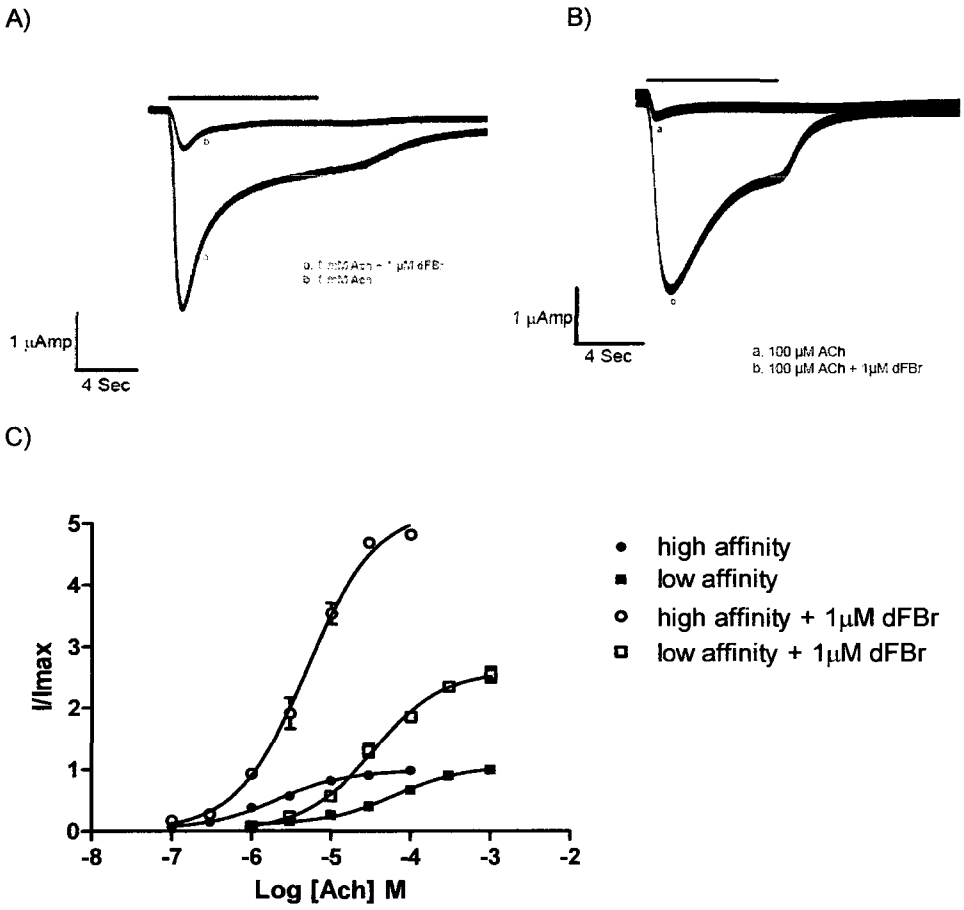


Figure 4.2: The effect of dFBr on $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ receptors.
$(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ receptors have high and low affinities for ACh respectively. Responses shown in A and B were obtained using Xenopus oocytes and two-electrode voltage clamp. Membrane potentials were clamped at -60 mV. Peak current amplitudes for each point were normalized to peak currents obtained in the presence of I_{max} for ACh without dFBr present. Data plotted in C were obtained from at least 4 different experiments on multiple oocytes. Data are tabulated in Table 4.2. A: $(\alpha4)_2(\beta2)_3$ receptors: Traces a and b were obtained from same oocytes and represent traces obtained at 1 mM Ach. B: $(\alpha4)_3(\beta2)_2$ receptors: Traces a and b were obtained from same oocytes and represent traces obtained at 100μM Ach C: EC_{50} values obtained from ACh responses obtained in the absence and presence of 1 μM dFBr. All dose-response curves, data points are mean +/- S.E.M. Each data point on all the four curves were reflects at least 4 repeated evaluations for each point on multiple oocytes for all the curves (n=4). Lines correspond to the best fit obtained with the Hill equation.

Table 4.2: EC_{50} for ACh activation, I_{max} and Hill slopes for responses obtained by ACh and by co-application of ACh with dFBr on High and Low affinity receptors. All values are mean +/- S.E.M.

	High affinity $(\alpha4)_2(\beta2)_3$	**Low affinity** $(\alpha4)_3(\beta2)_2$
EC_{50} (pM)	2.00 +/- 0.33μM	69.5 +/- 10.3μM
ACh Only		
EC_{50} (μM)	4.83 +/- 0.48μM	37.7 +/- 7.14μM
ACh + 1 μM dFBr		
Change in I_{max} resulting from co-application with 1 μM dFBr.	5.04 +/- 0.07	2.72 +/- 0.18
Hill slope	0.85 +/- 0.17	0.87 +/- 0.02
ACh only		
Hill Slope	1.25 +/- 0.15	0.97 +/- 0.16
ACh + 1 pM dFBr		

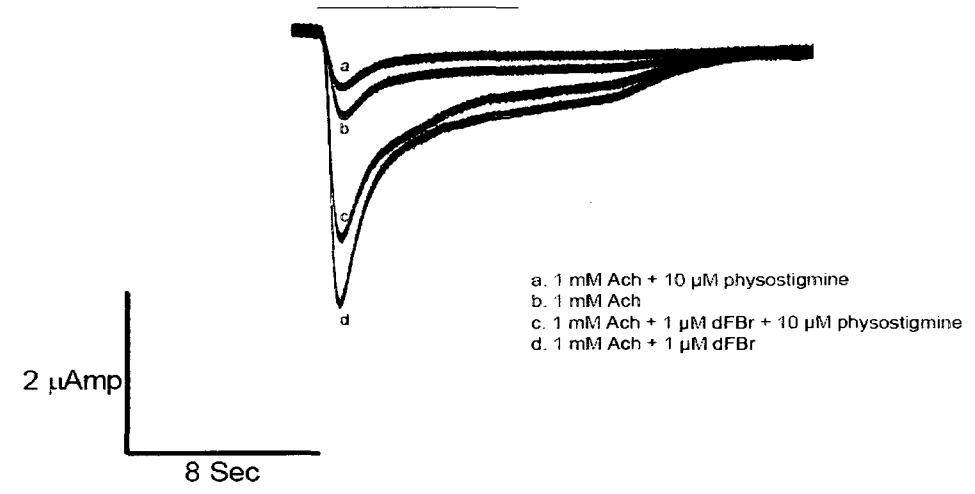
4.4.2. Physostigmine and dFBr:

Physostigmine is an acetylcholinesterase enzyme inhibitor which allosterically potentiates nAChRs (Maelicke et al., 2000). We compared dFBr and Physostigmine for their action as PAMs for α4β2 receptors.

Figure 4.3 shows the effect of physostigmine on α4β2 receptors expressed in Xenopus oocytes and stimulated with 1 mM ACh. Oocytes were injected with a 1:1 ratio of α4 and β2 subunits thus producing a mixture of high and low affinity receptors. A concentration of 10 pM physostigmine has been shown to be the concentration that provides maximum potentiation of α4β2 receptors expressed in Xenopus oocytes when co-applied with low concentration of ACh (1 μM) (Smulders et al., 2005). However when applied with higher ACh concentration, 10 pM Physostigmine appears to inhibit the response but does not

change the response kinetics (Figure 4.3A). When applied in combination with 1 μM dFBr, the dFBr potentiated response is also inhibited by the presence of physostigmine. To quantify this data, concentration response curves for ACh were determined in the presence of 1 μM dFBr, 10 μM physostigmine and a combination of both 1 μM dFBr and 10 μM physostigmine (Figure 4.3B) (n=4). Co-application of 10 μM physostigmine appears to shift the concentration response curve to the right, thus decreasing the EC_{50} from 36.8 +/- 8.75μM (ACh only) to 117 +/- 21.8 μM (ACh + 10 μM physostigmine). This represents a significant change in apparent affinity (P=0.0425). Hill slopes did not change significantly (P=0.9347).

A)



B)

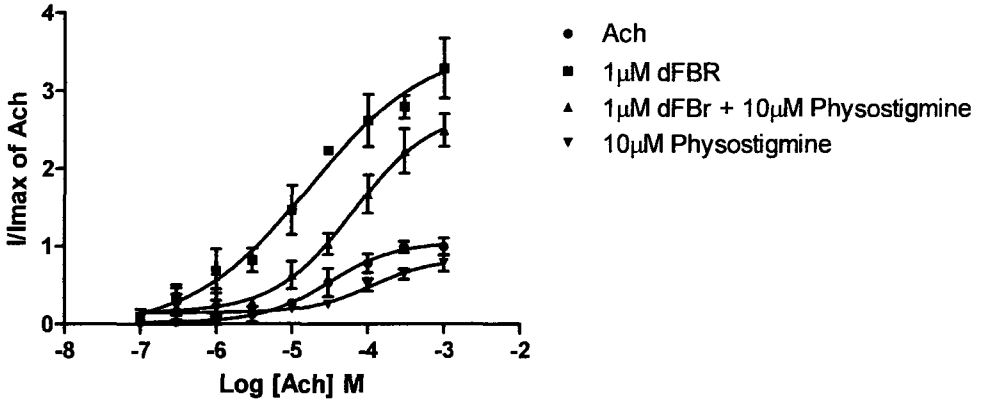


Figure 4.3: The effects of physostigmine and dFBr on α4β2 receptors.
Responses shown in A and B were obtained using Xenopus oocytes and two-electrode voltage clamp. Membrane potentials were clamped at -60 mV. Peak current amplitudes for each point were normalized to peak currents obtained in the presence of 1 mM ACh without dFBr or physostigmine present. Data are tabulated in Table 4.3. A: Responses obtained from Xenopus oocytes expressing α4β2. Membrane potential was clamped at -60 mV. Traces a, b, c and d were obtained from different oocytes and were normalized to trace obtained with 1 mM Ach. B. ACh concentration/response curve in the presence dFBr, Physostigmine and dFBr with physostigmine on human α4β2 receptors. All dose-response curves, data points are mean +/- S.E.M. Each data point on all the four curves were reflects at least 4 repeated evaluations for each point on multiple oocytes for all the curves (n=4). Lines correspond to the best fit obtained with the Hill equation.

Table 4.3: EC_{50} for ACh activation, I_{max} and Hill slopes for responses obtained by physostigmine, dFBr and by co-application of physostigmine with dFBr on mixed stoichiometry α4β2 receptors. The I_{max} was normalized to that obtained for 1 mM ACh on the same oocyte. All values are mean +/- S.E.M.

	EC_{50}	**I_{max}**	**Hill Slopes**
ACh	36.8 +/- 8.75μM	1.03 +/- 0.04	1.07 +/- 0.17
ACh + 1 μM dFBr	18.9 +/- 3.22μM	3.57 +/- 0.23	0.60 +/- 0.01
ACh + 10 μM Physostigmine	117 +/- 21.8 μM	0.78 +/- 0.11	1.11 +/- 0.21
ACh + 1 μM dFBr + 10 μM Physostigmine	59.8 +/- 7.89 μM	2.73 +/- 0.22	0.96 +/- 0.06

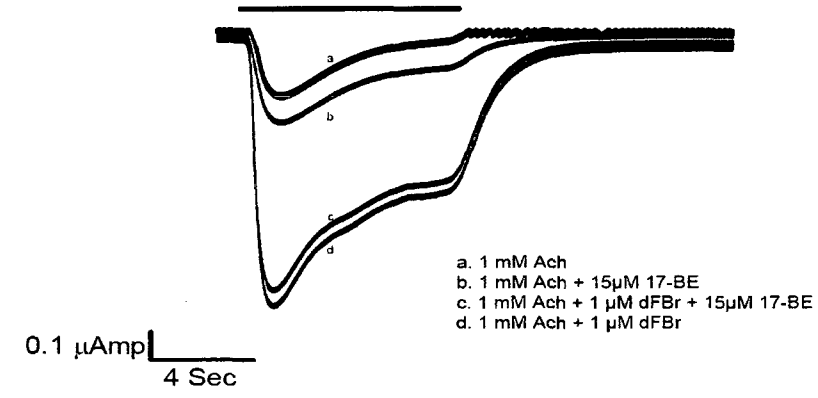
4.4.3. 17- β Estradiol and dFBr

17-BE is the first and only steroid molecule reported as a positive allosteric modulators of nAChRs. It was determined that the short extracellular c-terminal amino acid sequence (WLAGMI) of human α4β2 receptors is critical for potentiation by 17-BE (Paradiso et al., 2001). In this study, we compare the functional effects of dFBr and 17-BE on α4β2 receptors expressed in Xenopus oocytes.

Figure 4.4 shows the effects of co-application of ACh with dFBr, 17-BE and a combination of 1 μM dFBr and 15 μM 17-BE on human α4β2 receptors expressed in Xenopus oocytes (n=4). A concentration of 15μM 17-BE elicited maximum potentiation of human α4β2 receptors (Curtis et al., 2002). Oocytes were injected with a 1:1 ration of α4 and β2 subunits thus producing a mixture of high and low affinity receptors. Responses to 1 mM ACh appear slightly potentiated by 15 μM 17-BE, although much less then that by dFBr (Figure 4.4A). Response kinetics appears similar to un-potentiated responses although faster

rise times to peak are apparent. Combining both dFBr and 17-BE did not produce additive or synergistic effects but produced similar peaks amplitudes to dFBr alone. Quantification of this data in terms of changes in ACh concentration response kinetics yielded Figure 4.4B. 17-BE produced a slight change in EC_{50} similar to that observed for dFBr (Table 4.4). The changes in EC_{50} were not significant for either 17-BE or dFBr ($P>0.05$). Similarly to the effect of dFBr on Hill slopes, no significant change in Hill slopes is observed upon application of 17-BE ($P=0.2873$). The change in I_{max} produced by 17-BE was much smaller then dFBr but a significant 128% increase was observed ($P=0.0186$). This is in contrast to the 347% increase observed for dFBr ($P=0.0009$). Co-application of dFBr with 17-BE produced a significant decrease in Hill slopes to about 1/2 the un-potentiated response ($P< 0.0439$), however the change in EC_{50} was not significantly ($P= 0.3393$). The change in Hill slope may suggest a decrease in cooperativity between the ACh orthosteric sites as a result of the combined application of dFBr and 17-BE.

A)



B)

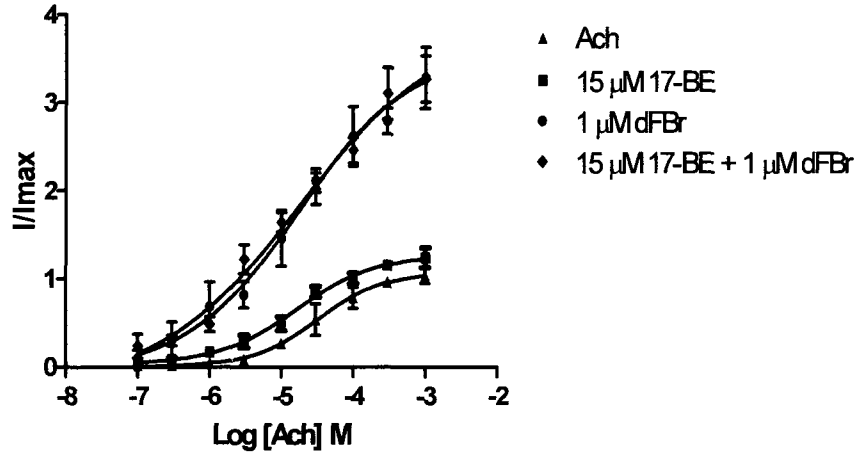


Figure 4.4: The effects of 17-BE and dFBr on α4β2 receptors.
Responses shown in A and B were obtained using Xenopus oocytes and two-electrode voltage clamp. Membrane potentials were clamped at -60 mV. Peak current amplitudes for each point were normalized to peak currents obtained in the presence of 1 mM ACh without dFBr or 17-BE present. Data are tabulated in Table 4.3. A: Responses obtained from Xenopus oocytes expressing α4β2. Membrane potential was clamped at -60 mV. Traces a, b, c and d were obtained from different oocytes and were normalized to the trace obtained from 1 mM Ach. B. ACh concentration/response curve in the presence 1 μM dFBr, 15 μM 17-BE and dFBr with 17-BE on human α4β2 receptors. All dose-response curves, data points are mean +/- S.E.M. Each data point on all the four curves were reflects at least 4 repeated evaluations for each point on multiple oocytes for all the curves (n=4). Lines correspond to the best fit obtained with the Hill equation.

Table 4.4: EC_{50} for ACh activation, I_{max} and Hill slopes for responses obtained by application of dFBr and 17-BE and by co-application of dFBr with 17-BE on α4β2 receptors. The I_{max} was normalized to that obtained for 1 mM ACh on the same oocyte. All values are mean +/- S.E.M.

	EC_{50}	**Imax**	**Hill Slopes**
ACh	36.8 +/- 8.75 μM	1.03 +/- 0.04	1.07 +/- 0.17
ACh + 1 μM dFBr	18.9 +/- 3.22 μM	3.57 +/- 0.23	0.60 +/- 0.01
ACh + 15 μM 17-BE	19.7 +/- 4.20 μM	1.32 +/- 0.09	0.78 +/- 0.08
ACh +1 μM dFBr + 15 μM 17-BE	25.2 +/- 6.69 μM	3.62 +/- 0.47	0.46 +/- 0.08

4.5 Discussion:

The discovery of dFBr and its establishment as an allosteric modulator of α4β2 receptors raises the question of whether dFBr truly represents a new class of modulator for this receptor with a unique mechanism or site of action or whether it is acting similar to another class of modulator (Kim et al., 2007). The modulators described here, physostigmine, Zn^{2+} and 17-BE, represent the key groups of allosteric modulators and have previously been shown to bind at distinct sites with specific mechanisms of action. We have examined the effects of all three modulators along with dFBr both independently and on co-application within the same assay system. The results of this study suggest that dFBr my be acting similar to either Zn^{2+} or 17-BE although differences in selectivity of high versus low affinity nicotinic receptors for dFBr and Zn^{2+} along with an observed additive effect of these agents on EC_{50} when co-applied would suggest that dFBr and Zn^{2+} may act via different mechanisms. The similarity of dFBr and 17-BE and the lack of any additive effect of these two compounds when co-applied strengthens the argument that dFBr and 17-BE may be

acting via similar mechanisms. This conclusion is useful in developing strategies to identify the dFBr binding site. Targeting of the putative 17-BE with mutagenesis and a determination of the effect of these mutations on dFBr potentiation will ultimately help determine if 17-BE and dFBr share a common binding site.

Zinc and dFBr: There is a clear indication that zinc and dFBr are working with each other to increase the apparent affinity of ACh for mixed (1:1) ratio expression of α4β2 receptors. As shown in Table 4.2. dFBr and zinc together significantly (P=0.05) increased the apparent affinity of ACh for α4β2 receptor more than that brought about by either Zinc or dFBr individually. The Hill coefficientalso decreases significantly (P=0.05) in presence of both dFBr and Zinc. This value of Hill coefficientis less than that obtained by application of dFBr or Zinc alone. The mechanism of zinc and dFBr maybe overlapping since zinc does not exert any significant effect on apparent efficacy in the presence of dFBr. The decrease in hill slope indicates increased cooperativity between the zinc and dFBr binding sites to stabilize the open receptor conformation induced by ACh. The cooperativity between dFBr and zinc indicate that each has a different binding site on α4β2 receptors.

Zinc and dFBr may share a similar mechanism of action but clearly have a different binding site, although this binding site may overlap with each other. The zinc potentiation site has been proposed to be present at the α4+/ α4- site of α4β2 receptors (Moroni et al., 2008), while other researchers have shown that the zinc-binding residues are be present at the β4+/α4- cleft of the α4β4 receptors (Hsiao et al., 2006). The putative dFBr binding site could also be present at β2+/α4- or the β2+/β2- cleft of the α4β2 receptors. Data obtained from this study would help target these clefts in α4β2 receptors for future site directed mutagenesis studies. Critical residues like D218 of β2 subunit and H195 of the α4 are important in binding zinc (Moroni et al., 2008). Such mutations will help identify the differences in the binding site of dFBr and zinc.

Physostigmine and dFBr: Physostigmine is the only one of the three PAMs which reduced the apparent apperant and apparent efficacy of ACh in presence and absence of dFBr in α4β2 receptors. As seen in table 4.3, Physostigmine reduced the apparent affinity of ACh and efficacy on α4β2 receptors. 10 μM physostigmine increases the apparent ACh EC_{50} from 36.8 μM to 117 μM. This amounts to about a 3.17 fold decrease in apparent affinity of ACh for α4β2 receptors in presence of physostigmine. The apparent ACh EC_{50} for α4β2 receptors in presence of 1 μM dFBr is 18.9 μM, which increased to 59.8 μM when 10 μM physostigmine is present. This indicates that there is a 3.16 fold decrease in apparent affinity of ACh in the presence of 10 μM physostigmine with the presence of 1 μM dFBr. The reduction in EC_{50} and hill coefficients by physostigmine indicates an inhibition of the action of ACh on the α4β2 receptors, irrespective of the presence of dFBr. The data from ACh EC_{50} experiments (Figure 4.3) supports the observation that physostigmine is directly inhibiting the actions of acetylcholine itself since the amount of decrease in apparent affinity of ACh for α4β2 receptors both in the presence and absence of dFBr is similar (3.17 to 3.16 fold without and with dFBr respectively). Therefore, physostigmine may be reducing the apparent ACh affinity on α4β2 receptors by a mechanism unhampered by the presence of dFBr. Thus, the effects of dFBr and physostigmine appear independent. These data support a model in which physostigmine and dFBr exert effects mediated by independent mechanisms and this mechanism supports the hypothesis that there are different binding sites for physostigmine and dFBr. Physostigmine is a partial agonist for α4β2 receptors and it is thought to bind the receptor at an ACh equivalent agonist recognition sites (Smulders et al., 2005). The inhibition of α4β2 receptor by physostigmine is non-competitive via ion channel block. (Smulders et al., 2005). Additionally, on the torpedo nAChRs physostigmine binds to the FK-1 antibody site around Lys-125, which is also the an low affinity ACh binding site on the

torpedo nAChRs (Schroder et al., 1994). Physostigmine and dFBr have a different binding site on α4β2 receptors and independence of action.

17-β-Estradiol and dFBr: 17-BE which potentiates α4β2 receptors is closely related structurally to Progesterone which is a non competitive inhibitor of α4β2 receptors (Arias et al., 2006). Progesterone does not bind to the 17-BE potentiation site (Paradiso et al., 2001). The chemical structure of dFBr and 17-BE are very different. dFBr is an indolic alkaloid derivative (Peters et al., 2004) whereas the structure of 17-BE is cholesterol based (Figure 4.5);

Data from the ACh EC_{50} experiments (Figure 4.4) shows that the apparent affinity of ACh was similar when 1 μM dFBr and 15 μM 17-BE were applied individually or together on α4β2 receptors. The efficacy value for dFBr + 17-BE on α4β2 receptors is the same as the efficacy value of dFBr by itself. There is no significant ($P > 0.05$) enhancement or decrease in either apperant affinity or apperant efficacy for dFBr with 17-BE compared to dFBr alone. There is no interdependence in the action of dFBr and 17-BE on each other. 17-BE exerts a smaller effect of potentiation on α4β2 receptors which was masked when dFBr is present. dFBr exerted a larger potentiating effect on α4β2 receptors compared to 17-BE. There is no synergy between 17-BE and dFBr hence the mechanism of potentiation by dFBr and 17-BE may be overlapping as seen for zinc. However the differences in the chemical structural of dFBr and 17-BE makes it unlike that these two ligands would bind to the same site. However the binding site for dFBr and 17-BE may also possibly have some overlap. The data with dFBr and 17-BE gives a clear direction to future site directed mutagenesis studies that would target the four residues (AGMI) in the C-terminal of the α4 subunit C-terminal which are know to bind 17-BE (Paradiso et al., 2001).

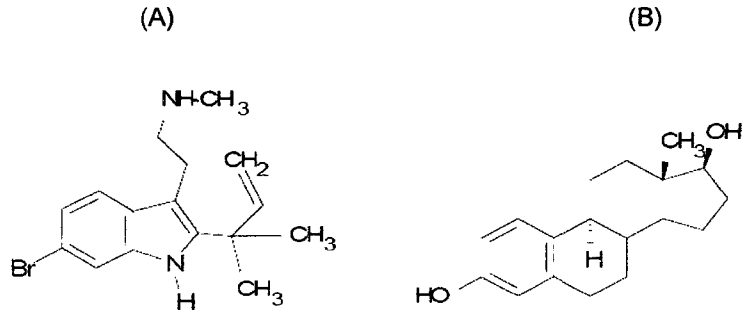


Figure 4.5: Structure of: Desformylflustrabromine (A); 17-Beta-Estradiol (B).

4.6 Conclusion:

The experimental evidence indicates that the action of dFBr is independent of the presence of Physostigmine, zinc and 17-BE. The data obtained indicate that Physostigmine, zinc and 17-BE applied with dFBr failed to enhance apparent efficacy compared to that produced by dFBr alone. Only physostigmine reduced apparent apperant and apparent efficacy of ACh on α4β2 receptors in presence and absence of dFBr. Interestingly the proportion of reduction of apparent efficacy and apparent apperant by Physostigmine was not influenced by the presence or absence of dFBr. The presence of physostigmine reduced the effective ACh concentration which was uninterrupted by dFBr. Zinc and 17-BE did not induce any additional potentiation with dFBr on α4β2 receptors. Zinc, 17-BE and dFBr may share a similar mechanism of potentiation action on these receptors. A synergistic decrease in the Hill coefficient for dFBr/Zinc co-application might suggest some overlap in the binding domains between dFBr and zinc. 17-BE produced no additive effects on apparent affinity or efficacy indicating overlapping or similar mechanisms. The structural dissimilarity between 17- BE might suggest they act by similar mechanisms but via different binding sites but that is not discernable from our data.

Identification of a binding region for a new drug such as dFBr can be challenging and must rely on clues from experiments such as those described here. The data in this chapter

supports further study of the putative binding domains for zinc and 17-BE and narrows the initial scope of mutagenesis studies to these areas. Zinc is thought to bind at the subunit interfaces that do not contain acetylcholine binding sites therfore this region of the receptor would be an excellent starting point for mutagenesis. Since 17-BE also shows similarities to dFBr the C-terminal end of the α4 subunit should also be explored. A particularly interesting result would be identification of overlap between zinc and dFBr. Zinc is not a particularly good lead for drug development but identification of an alternate ligand for the zinc potentiation site would be an exciting development as it would provide a starting point for further exploring this site for drug development. Mechanistic experiments in the future will help determine the extent of similarity on mechanisms between these ligands.

The data obtain supports our hypothesis that the effects of dFBr are due to its binding at a unique binding site that is different from the binding site for other α4β2 receptor allosteric modulators, i.e., physostigmine, zinc or 17-BE. These results show that dFBr is a new class of positive allosteric modulator for α4β2 subtype of neuronal nicotinic receptors.

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CHAPTER 5: CONCLUSION AND FUTURE DIRECTION

5.1 An overview

Nicotinic acetylcholine receptors (nAChRs) are widely distributed in the peripheral and central nervous system, and play a major role in mediating various physiological processes. It is imperative that novel ligands be developed which selectively target these receptors. Selective ligands could be useful clinically in diseases and pathological conditions where nicotinic tone in the CNS is altered. While many ligands have been developed which target α4β2 receptors, these ligands typically act on the receptors via the orthosteric site, which is the ACh and nicotine binding site. The orthosteric site is well conserved across all subtypes of nicotinic receptors and hence subtype selective ligands have been elusive. Orthosteric agonists and partial agonists tend to desensitize and/or produce up regulation of α4β2 receptors expression (Gentry and Lukas, 2002). The development of allosteric ligands may help overcome some of these obstacles. Since allosteric sites are likely to be less conserved across nicotinic subtypes, these ligands may display greater subtype selectivity. Additionally, since allosteric ligands do not activate receptors but instead modulate the responses to endogenous ligands, control over activation remains under the control of pre-synaptic transmitter release. This may also make allosteric ligands less prone to producing changes in receptor expression.

This thesis focuses on a novel type of positive allosteric modulator targeted at the α4β2 nicotinic receptor subtype. While this work has not specifically focused on receptor selectivity, this is an important long term goal of the research. Before that goal can be accomplished detailed information regarding optimization of the dFBr pharmacophore and its interaction with the receptor is required. Chapter 2 of this thesis evaluated the effects of a synthetic dFBr to confirm that this compound was capable of producing the effects reported for the natural product. Chapter 3 expanded on this work to identify key structural features of

dFBr that contribute to its potentiating effects on α4β2 receptors as well as its inhibitory effects on α4β2 and α7 receptors. Chapter 4 addressed the issue of mechanism of action, possible binding site location and similarity to other PAMs. Taken as a whole, these data set the stage for a second phase of research that will explore in greater detail the receptor regions responsible for the binding site involved in dFBr potentiation and optimize the dFBr drug class as a selective α4β2 PAM. While it is expected that dFBr will form the basis of a new, therapeutically useful agent, understanding the effects and interaction of this compound will also contribute towards an improved understanding of the nicotinic receptor family and, by extension, ligand gated ion channels in general. Among the contributions expected from this work are:

1) Identification of receptor mechanisms: Conventional agonists, antagonists and partial agonist act on the nicotinic receptors via interaction with the orthosteric site. Study of allosteric modulation may reveal new features of the mechanisms underlying receptor activation.
2) Identification of novel allosteric binding sites on neuronal nicotinic: Neuronal nicotinic receptors may contain multiple allosteric binding sites in addition to the orthosteric, acetylcholine site. Using novel ligands as molecular probes, putative allosteric sites on nicotinic receptors can be found and studied. Experimental methods like site directed mutagenesis, chimeric constructs and the substituted cysteine accessibility method (SCAM) can be utilized to pinpoint the amino acid residues in the allosteric binding site and their involvement in binding of allosteric ligands.
3) Discovery of novel allosteric modulatory ligands for neuronal nAChRs: One purpose of basic pharmacological research is to produce new lead molecules that can be developed into novel drug therapies. The discovery of novel allosteric modulators is the first step in developing therapeutic ligands that selectively target nAChR subtypes. Since the large majority of nAChR modulators currently available are non-

selective, the development of subtype selective ligands will facilitate identification of the role of select subtypes in disease processes.

5.2 Conclusions:

1) **Synthetic desformylflustrabromine is a unique positive allosteric modulator of α4β2 subtype of neuronal nicotinic receptors.**

The ligand-gated ion channel super-family (LGICS) includes a wide variety of ion channel receptors including multiple nAChRs, 5-HT_3Rs, GABA (A) and (C) receptors and glycine receptors. All of these proteins, including a closely related acetylcholine binding protein (AChBP) are allosteric proteins. It is thus not surprising that multiple ligands acting at distinct binding sites can produce alterations in receptor function. For example, the GABA (A) receptor is modulated by a number of compounds that bind to allosteric sites on the receptor. These modulators include valuable therapeutic drug classes including Benzodiazepines (BDZ) and Barbiturates. These allosteric modulators of the GABA (A) receptor are commonly prescribed clinically as anti-depressants, anxiolytics and anaesthetics (Whiting, 2003). Given the structural similarity of all members of the LGIC family it is likely that all members contain allosteric modulatory binding sites. For the nicotinic receptors the only clinically used modulator is galantamine although this compounds primary site of action in the CNS appears to be inhibition of acetylcholinesterase while its direct modulatory effects on nAChRs are likely secondary. No modulatory agent similar to BDZ or Barbiturates has been developed for clinical use in modulating nicotinic receptor activation. dFBr represents a unique positive allosteric modulator of nAChRs and our goal is to develop it into a therapeutically useful drug. To get to this endpoint, a clear understanding of its interaction with the nAChR and its mechanism of action is necessary.

Our original hypothesis for explaining the mechanism of action of dFBr was based on comparisons with the action of BDZs on the GABA (A) receptor. Chlordiazepoxide, a BDZ

drug, allosterically modulates the activation of GABA (A) receptor by GABA (Choh et al., 1977) by increasing the apparent affinity of the neurotransmitter (GABA) for the receptor (Choi et al., 1981). Using the action of BDZ on GABA (A) receptor as a model, we proposed that dFBr might allosterically modulate the α4β2 subtype of neuronal nAChRs by increasing the apparent affinity of acetylcholine for the receptor. Since early studies of dFBr used compound obtained from a natural source, our first step was to confirm that the proposed active component was indeed dFBr (Sala et al., 2005). Synthetic dFBr does not contain any potential contaminating analogues or metabolites and we demonstrated in Chapter 2 that this compound was capable of potentiating ACh induced responses at α4β2 receptors. However, the EC_{50} for synthetic dFBr was over 10X greater then that obtained from natural sources. We also discovered a previously unidentified inhibitory effect of dFBr evident at concentrations greater then 10μM. This effect was likely observed only in the synthetic compound due to improved solubility of the synthetic HCl salt of dFBr compared to the less soluble natural product as well as increased purity of the product. Similar to the natural form of dFBr, the synthetic compound was selective for heteromeric α4β2 receptors versus homomeric α7 receptors (Sala et al., 2005). For α7 receptors we observed inhibition but no potentiation. The primary outcomes of the data presented on synthetic dFBr in Chapter 2 were confirmation of the potentiating nature of dFBr, the discovery that dFBr was also capable of inhibiting the α4β2 and α7 receptors at concentrations greater then 10□M and the ability to synthesize sufficient quantities of dFBr for further studies. This enabled us to move on to the work of identifying the key structural components of dFBr responsible for these actions that is presented in Chapter 3.

2) **Different functional groups in the chemical structure of desformylflustrabromine are responsible for the potentiation versus inhibition of acetylcholine induced responses on α4β2 receptors.**

We evaluated 16 synthetic analogues of dFBr, two of which occur naturally. As described in Chapter 3, four of the 16 synthetic analogues tested potentiated ACh induced responses on α4β2 receptors. Similar to dFBr, these analogues potentiated responses at low concentrations and inhibited at higher concentrations. The remaining 12 analogues, including two compounds that occur naturally with dFBr, inhibited ACh induced responses of α4β2 receptors but did not potentiate. On α7 receptors, all of the 16 analogues, including dFBr itself, inhibited ACh induced responses.

Different functional groups in the chemical structure of dFBr and its analogues were identified as responsible for their potentiating and inhibitory actions. For potentiation, two key groups appear important: The geminal dimethyl group and the bromine attached to the indole. Removal of the gem dimethyl eliminates potentiation and removal of the bromine significantly reduces the EC_{50} for potentiation. Modification of other functional groups had little effect on potentiation but had larger effects on inhibition of α4β2 receptors. The different structure/activity relationships for potentiation and inhibition support the hypothesis that inhibition and potentiation represent two distinct mechanisms and sites of action of dFBr on the receptor. This is an important determination since it suggests that dFBr can be optimized to obtain a compound that will display potentiation but no inhibition. This would likely result in a new compound similar to dFBr but with substantially enhanced efficacy. In future studies, further detailed exploration of the precise requirements of these functional groups will be accomplished through addition of a variety of modified groups at these positions. This "reconstruction" phase will produce not only enhanced efficacy but also increased selectivity. While this thesis does not specifically address the broad issue of receptor selectivity, the data discussed in Chapter 3 can be used in future studies to identify and optimize the selectivity of dFBr for specific receptor subtypes. Identification of the specific binding domain for dFBr on α4β2 receptors and the key pharmacophoric features of dFBr are critical to this

next step. In Chapter 4, we began to address the binding site location by determining if it is likely to be similar or different then other PAMs.

3) **Comparison of dFBr and other nicotinic PAMS reveals similarities in potentiating mechanisms and possible locations of the potentiating binding site for dFBr.**

Along with dFBr, physostigmine, Zn^{2+} and 17-BE are also known to potentiate α4β2 receptors. We therefore performed experiments comparing the pharmacology and induced effects of dFBr and other positive allosteric modulators of the α4β2 receptor. The initial studies presented in Chapter 4 explored the effects of these compounds on ACh induced responses on α4β2 receptors using our assay system. Physostigmine is an acetylcholinesterase inhibitor which potentiates α4β2 receptors in the presence of low concentrations of ACh. Zn^{2+} is a divalent cation with potentiates only the low ACh affinity stoichiometry $(\alpha4)_3(\beta2)_2$ receptor (Moroni et al., 2008). 17-BE potentiates α4β2 receptors by binding to the short extracellular C-terminus of α4 subunit in α4β2 receptors (Paradiso et al., 2001). All four compounds were evaluated independently and in combination with dFBr.

Physostigmine reduced affinity of α4β2 receptors for ACh and increased the response to ACh concentrations less then 10 mM but inhibited responses when co-applied with ACh at higher concentrations. Thus, unlike the remaining three compounds tested, physostigmine did not alter the apparent efficacy of ACh. This is in contrast to dFBr which alters ACh affinity only slightly but greatly alters apparent efficacy. When co-applied, dFBr and physostigmine produce a result that appears to be the sum of the changes they induce independently. The additive nature of this interaction suggests that the two compounds produce two different effects through different basic mechanisms.

Zn^{2+} has been shown to potentiate low affinity α4β2 receptors but not high affinity receptors. This is in constrast to dFBr which shows a slight preference for potentiation of high affinity receptors. This difference in subunit selectivity suggests at least differences in

binding site characteristics for the two compounds. Both compounds produce slight decreases in the ACh EC_{50} and increase the apparent efficacy. When co-applied with dFBr, Zn^{2+} does not produce any increase in the apparent efficacy compared to dFBr alone. This lack of additional change in apparent efficacy might suggest that dFBr and Zn^{2+} act by similar mechanisms, but the decrease in EC_{50} suggests independent mechanisms. These data could be consistent with a hypothesis that dFBr and Zn^{2+} produce similar effects but through possibly different binding sites. One explanation would be binding of Zn^{2+} and dFBr to different subunit interfaces such as α^+/β^- versus β^+/α^-. This would also explain the differences in selectivity for subtypes with different subunit stoichiometries.

17-BE produced similar, although much smaller changes in EC_{50} and apparent efficacy when compared to dFBr, however, unlike physostigmine and Zn^{2+} no additional changes in either parameter were observed when the two compounds were co-applied. While this lack of any cumulative effect is not definitive, it does suggests similar mechanisms or sites of action for 17-BE and dFBr. This hypothesis can be tested by utilizing single channel techniques to determine if dFBr shows stabilization of open state conformation (as seen for Zn^{2+}) or produces bursting (as seen for 17-BE). Site directed mutagenesis of the C-terminal region would be helpful in confirming if the C-terminal binding site for 17-BE is shared, or overlaps, the dFBr site

5.3 Future direction:

dFBr is a lead molecule of natural origin which has been validated as a positive allosteric modulator for the α4β2 subtype of neuronal nAChRs (Kim et al., 2007). Here we have reported the results from experiments testing the action of synthetic dFBr, a phase 1 SAR study and identification of a putative binding region on a4b2 receptors. Additional studies will be necessary to further develop the dFBr pharmacophore, confirm the dFBr binding site and model the interaction of dFBr with the receptor. Ultimately, the goal is to

establish dFBr analogues as clinically useful drug candidates. These studies will also contribute to understanding α4β2 receptor mechanisms. Recommended future studies are:

1) Synthesis and evaluation of additional analogues of dFBr. This will be necessary to optimize the dFBr pharmacophore and further develop the structure-activity relationship.
2) Confirm the location of the dFBr binding site. Two possible locations have been proposed for the dFBr binding site location based on the data obtained in Chapter 4; the β^+/β^- cleft and the C-terminal 17-BE site. Exploration of both regions using site directed mutagenesis will confirm or refute these hypotheses. For this purpose, alanine scanning mutagenesis (ASM) can be used where each amino acid in a chosen region of the protein is mutated sequentially to alanine (Cunningham et al., 1989). An alanine substitution of an amino acid residue involved in dFBr binding to the protein will lead to a significant change in receptor function. Potentially important residues identified by alanine scanning can later be mutated to more similar amino acids to determine specifics of the interactions at these positions; i.e., a Phy (F) to a Typ (W). Another commonly used technique for identification of the binding site on receptor proteins is the substituted cysteine accessibility method (SCAM). In this technique residues within the binding domain are mutated to cysteine (Cys) residues then subject to modification by sulfhydryl reagents (Wiltfong and Jansen, 2009). Cys residues that are resistant to modification in the presence of ligand but modified in the absence of ligand are presumed to be protected by the ligand because they are located in the binding site. This technique provides important information about location of the binding site but does not provide details about specific amino acid/ligand interactions. These interactions are probed with traditional site directed mutagenesis approaches.
3) Mechanism of action: Determination of mechanism of action will require single channel studies. Single channel recording is the most direct method available for obtaining detailed and precise information about the kinetic behaviour of ion channels. The goal of

these studies will be to determine the precise nature of the dFBr effect compared to other modulators. By recording single channel openings and closings over time, it is possible to identify changes in stability of open versus closed states, changes in singe channel conductance and changes in the latency of channel opening after application of dFBr.

4) Animal models: The effect of dFBr in animal models for specific diseases can also be studied. Genetically-engineered mice have been developed that exhibit both behavioural characteristics of Alzheimer's dementia and protein-derived plaques like those which appear in the brains of patients with Alzheimer's disease (Hsiao, 1998). A genetically engineered mouse showing excessive grooming and other anxiety-like traits have also been developed (Korff and Harvey, 2006). Such mice are a good model for obsessive compulsive disorder. Mouse models with cognitive deficits have also been developed (Fisch, 2007). dFBr can be directly administered to such animals. Mouse models for fragile X syndrome, Rett syndrome and other disorders associated with autistic-like behaviour have been developed (Moy and Nadler, 2008). If dFBr could relieve symptoms in mice this might indicate a potential therapeutic value of dFBr for neurological disorders.

Pre-clinical Studies:

From this point onwards the research involving dFBr will follow the path which is taken by all drugs are made to be market ready (Remington, 1995).

1) Toxicity studies: Toxicology studies are needed to evaluate the safety of the drug. It is likely that a drug candidate targeting a specific receptor or enzyme on a certain cell type may have a beneficial outcome in providing treatment for certain disease or condition. However, such a drug may also interact with other proteins on different cell types in other organs and cause unintended harm due to these interactions. A clear example of this is

the drug thalidomide. Thalidomide was developed during the late 1950s and early 1960s as a sedative-hypnotic (Diggle, 2001). It was prescribed to women during pregnancy for morning sickness and as a sleep aid. However, the safety of the drug was not evaluated properly before being release to patients for its use. This resulted in women having children with severe malformities like phocomelia. Nearly 10,000 cases of such birth were reported the world over because expectant mother were prescribed thalidomide during pregnancy (Diggle, 2001). The racemic mixture of Thalidomide contains both the left (s) and right (r) handed isomers in equal amounts. While the right handed enantiomer of thalidomide is effective against morning sickness, the left handed enantiomer is a potent teratogen which causes birth defects (Eriksson et al., 2001). The two enantiomers of thalidomide interconvert in vivo, hence when pure r-thalidomide or s-thalidomide is given to humans both isomers can be found in the serum (Eriksson et al., 2001). The mechanism of action of thalidomide is thought to be by its ability to intercalate into DNA in G-C rich regions (Stephens et al., 2000). Since then, thalidomide has been approved by FDA in 1999 as an agent for the treatment of multiple myeloma because of its anti-angiogenesis action (Barlogie et al., 2001).

2) Formulation development: After the characterization of physical and chemical properties of a lead molecule such as dFBr, a suitable formulation has to be developed for its administration. A formulation is designed based on properties, such as dissociation constant (pKa), melting point, solubility, morphologic structure (crystalline / amorphous) and partition coefficient among many others (Remington, 1995). An ideal molecule will lend itself to be developed in multiple dosage forms (Remington, 1995). Orally administered drugs (Tablet, capsule, powder) are suitable for most patients (Miller et al., 2007) except in cases where the patent is non-responsive (e.g., unconscious). A liquid dosage form (monophasic - syrup or biphasic – suspension and emulsion) is most conducive for administration to young patients: children and infants (Breitkreutz and

Boos, 2007). Even though injections represent a different route of administration, physio-chemically it is liquid or in some cases a powder (Remington, 1995). A drug in powder form can be developed in to creams and ointments for tropical or systemic use by using an appropriate base (Remington, 1995). Finally a drug can also be developed into aerosols and suppositories (Remington, 1995). A suitable dosage form has to be developed for a drug in order for it to be available for actual patients for use (Remington, 1995).

3) Pharmacokinetic parameter analysis: after a drug is developed in a suitable dosage form it has to be evaluated for its pharmacokinetic parameters. The pharmacokinetics parameters of a drug are largely influenced by its bio-chemical properties and its dosage form design (Remington, 1995). Four important criteria essentially make up the pharmacokinetics of a drug are:

   i) Absorption – the route of administration may determine absorption of a drug. The bioavailability of the drug is based on the rate of absorption. When a new drug is being developed, it needs to be determined if the drug undergoes first pass metabolism. If it does then is the metabolite active? If the metabolite is active then the drug is said to be a pro-drug (Hosokawa, 2008).

   ii) Distribution – The entry of the drug in the general circulation is called distribution (Katzung, 2004). It depends on factors such as plasma binding, lipid solubility of the drug and if it can cross various selectivity membrane of the body such as the blood-brain barrier or the placental membrane in pregnant women.

   iii) Biotransformation / metabolism – most drugs are metabolized in the liver, metabolically the most complex organ of the body (Goodman et al., 2008). The purpose of drug metabolism is to convert the drug into a form such that it can be easily excreted form the body (Goodman et al., 2008). Usually drugs undergo oxidation in the live by the cytochrome P450 enzyme system (Xu et al., 2005). In

cases where the drug is hydrophobic it undergoes processes such as, hydroxylation, gluconuride conjugation, sulfation, phosphorylation or methylation to produce water soluble metabolite (Xu et al., 2005).

iv) Elimination – Renal excretion is the most common way of drug elimination followed by sweat glands and stools (Goodman et al., 2008). In some cases drugs can also be eliminated orally through saliva or through maternal lactation process (Goodman et al., 2008).

4) Clinical trials: The steps in the process of drug development from here on are known as clinical trials. The preclinical trials both in vivo and in vitro help set the parameters for clinical trials for the drug (Goodman et al., 2008). The preclinical tests help decide if it is safe to give the drug to humans and what the toxic dose is. For accurate assessment of the result from the clinical studies a double blind study with and without placebo are carried out. In phase-1 clinical trials small group of healthy volunteers are chosen. Once the safety of the drug is established in this group, small group phase-2 trials can be conducted. In phase-2 trials a small number of patients in which the drug is supposed to be effective are chosen. The effectiveness and safety of the drug is established at the end of phase-2 trials (Goodman et al., 2008). In phase-3 clinical trials, large group of patients are included using both placebo and comparison standards. A large amount of safety and efficacy data is obtained analyzed and presented to the authorities for approval of the drug for general marketing. Once the drug is released into the market, the performance of the drug is continued to be evaluated for its efficacy and safety in vary large numbers of patients. This monitoring is often referred as phase-4 clinical trials. Additionally, clinical trials monitoring the drug in small focused group of individual such as geriatric patients, patients of certain racial make-up is carried out in order the access the safety and effectiveness of the drug in such groups.

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